ENZYMATIC REACTIONS IN MICROEMULSIONS AND MONOLAYERS

By

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To

My Parents, Brothers and Wife

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ENZYMATIC REACTIONS IN MICROEMULSIONS AND MONOLAYERS

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Esterification reactions are of great interest to the food and cosmetics industries for the synthesis of emulsifiers, texturizers, flavors and fragrances. In this research, esterification reactions have been carried out in water-in-oil microemulsions, monolayers and foams.

The esterification of glycerol and fatty acid in the presence of lipase was carried out in a water/diethylhexyl sulfosuccinate (AOT)/hydrocarbon microemulsion system which provides a large interfacial area for reaction to occur. The various parameters like oil chain length of continuous phase, unsaturation of fatty acid, chain length of fatty acid, water/AOT ratio and oleic acid/glycerol ratio are important for esterification. HPLC analysis of samples from microemulsions showed the presence of mono- and diglycerides. The average size of reacting microemulsion droplets was measured by the QELS technique.

The major obstacle for commercial use of traditional microemulsion systems based on surfactants like AOT and CTAB is very low substrate concentration in the oil phase (<6 % by weight). This limits the economical viability for process development. A new microemulsion system was developed where the oil water interface was stabilized by oleic acid (a substrate), Na-oleate and t-butanol. This microemulsion solubilizes a large amount of aqueous phase (40-45 % by wt.) and has a high substrate concentration of oleic acid in oil phase (20 % by wt.). The structure of this microemulsion was characterized by PGSE NMR which showed the presence of reverse micelles. Mono- and diglycerides but no triglycerides were the products from this microemulsion system.

Monolayers of oleic acid and Na-oleate were formed on a subsolution of glycerol and water. The enzyme solution was injected under the compressed monolayer to initiate the reaction. The products from monolayers contained diglycerides and triglycerides. The formation of triglycerides in monolayer experiments is believed to be due to the favorable orientation and longer residence time of products at the air-water interface compared to the oil/water interface in microemulsions.

Foams were generated by blowing air through a solution of glycerol, water, enzyme and stearic acid. HPLC analysis of the foam after reaction indicated the presence of diglycerides and triglycerides similar to those in monolayers.

CHAPTER 1
INTRODUCTION

1.1 Overview

Esterification reactions are technologically significant because of their importance to several industries related to foods cosmetics and coatings

This chapter deals with background information related to fatty acid esterification, lipase catalysis, types of media for lipase catalyzed reactions, microemulsions, and lipase catalyzed reactions in microemulsions Important and current literature related to these topics will be presented discussed, and cited in the following sections of this chapter.

1.2 Esterification

1.2.1 Definition

A reaction between a carboxylic acid and an alcohol to produce an ester and water is defined as an esterification reaction. This reaction is reversible (see figure 1.1) and the opposite reaction is called hydrolysis Other reactions related toesterification include inter-esterification or acidolysis, involving reactions between esters and free fatty acids, alcoholysis, where the reactants are esters and alcohols, and trans-esterification, where different esters are reacted All three reactions involve the breakage and/or reformation of ester bonds.

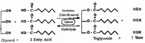


Figure 1 1 Schematic representation of esterification reactions.

1.2.2 Industrial Applications

Esterification reactions have applications in a number of food and cosmetic related industries. For example, most cooking oils contain primarily triesters of glycerol (a three-carbon tri-hydroxyl alcohol) and fatty acids called triglycerides. The physical properties of each oil, like flavor and color, are directly related to the composition of triglycerides and the types (saturated or unsaturated) and sizes of fatty acid groups contained in them [1]. The types of fatty acids present also dictate the nutritional value of the fat. For example, polyunsaturated fats, those containing many unsaturated fatty acid groups, are less likely to promote high blood cholesterol levels [2], and some unsaturated fatty acids, such as linoleic acid, are essential to the human diet [3]. However, the presence of large unsaturated fatty acid content promotes the onset of rancidity, which occurs due to peroxidation [1]. Thus, the presence of desired triglycerides in oil, produced by esterification, trans- or inter-esterification is strongly desired. Moreover, cheaper abundant oils such as palm oil can be converted into less abundant, more expensive oils such as cocoa butter by changing the fatty acid chains of triglycerides. On a further extreme, new oils containing the desired properties can be "tailor-made" by mixing specific triglycerides produced through esterification.

Other products of esterification like monoglycerides, which are monoesters of fatty acid and glycerol, are important (edible) emulsifiers found in foods such as cake, ice cream, and margarine [1], and in many cosmetic creams and lotions [4]. Monoglycerides are important intermediates in the food industry for synthesis of acetostearins, lactostearins, and mixed triglycerides [1]. Fatty acid esters of other polyols, such as ethylene and propylene glycols, also are of extreme importance in the cosmetic industry as emulsifiers and texturizers [4]. The properties and uses of mono-, di-, and triesters are significantly different; thus, catalysis by enzymes may be desirable to control the selectivity of esterification.

Fatty acid esters of sugars and sugar derivatives have received attention in recent years as useful surfactants/emulsifiers. Specific uses include food emulsifiers and low calorie fat substitutes, emulsifiers in cosmetics and medicines, surfactants in detergent formation and enhanced oil recovery (due to its biodegradability), and components of coating materials such as paints and varnishes [1,5].

Fatty acid esters of monohydroxyl alcohols also have a variety of uses. Esters of short-chained fatty acids as flavor and fragrance ingredients are common components of foods and cosmetics. For example, the ester of butyric acid and ethanol produces the fragrance of strawberries. Also, through catalyzation by enzymes, esterification and related reactions can be used for enantiomeric separation of racemic mixtures of alcohols on a preparative scale [6,7], which is an important process step in pharmaceutical syntheses. In addition, ethyl esters of fatty acids have been investigated as substitutes for diesel fuel [8].

1.2.3 Methods of Esterification

Triglyceride oils are derived from natural products, such as cottonseed oil and animal fat, through extraction driven by high pressure, temperature or lipophilic solvents (e.g., ethers) [3]. The oils are refined to remove impurities that cause discoloration, odor, and foaming. Such impurities include phospholipids, free fatty acids, aldehydes, ketones, and pigments. Treatment with hot alkali under agitation causes free fatty acids to saponify and separate from the oil. Other refining steps include bleaching with adsorbents (to remove pigments and soaps/alkali left from saponification) and deodorizing with vacuum steam distillation (to remove aldehydes and ketones), often known as the Wesson Method. Oils are hydrogenated to convert unsaturated fatty acid groups of triglycerides to saturated species. This conversion increases the melting points of oils and lessens their susceptibility to peroxidation and, hence, rancidity. This process is carried out by catalysis over nickel at high temperatures (100-200°C) and under vacuum. Another common refining step is winterization, where refrigeration over an extended period causes solidification of triglycerides having higher melting temperature. The liquid fraction could then be used to make cooking oil while the solid could be used in the manufacture of shortenings. The detailed information on the refining of oils and the specific production of margarines, shortenings, and other food materials is given elsewhere [1,3].

The modification of oils through cleaving and reforming ester bonds is undertaken for various reasons, as discussed above. Trans-esterification and inter-esterification, directed by high temperatures and/or acid/base or metal catalysis, are the most common procedures [1]. As an example, oils are mixed with fats containing large saturated fatty acids. After catalyzing ester interchange to an oil, one can alter the properties of the oil by changing the ratio of trisaturated to triunsaturated fats, which affects the oil's plasticity behavior, but such catalysis is not common. In the last decade, the use of lipases as biocatalysts was a major thrust of research due to their specificities and ability to react at ambient temperatures and pressures. This will be elaborated below.

In the first half of the century, mono- and diglycerides were normally derived from hydrolysis of oil triglycerides or the alcoholysis of fats by glycerol. These processes must be conducted at high temperatures and pressures using metal catalysts and are often limited in production rate and conversion by the poor solubilization of the former (oils or fats) into the latter (glycerol). A drawback to this type of process is its poor selectivity. Nowadays, most food companies employ the process of molecular distillation with steam to recover monoglycerides from oils [9]. But many high temperature processes often darken the color of product due to formation of by product. Therefore, in order to reduce operating costs and the by-product formation, enzymatic hydrolysis of fat has been investigated in the past decade.

The esterification between glycerol and fatty acid has been employed industrially for production of glycerides using a catalyst (most commonly, sulfonic acid) at high temperatures and subatmospheric pressures [1]. The extent of esterification is quite sensitive to the water content (water removal is necessary) and the process requires agitation and/or the addition of expensive organic solvents (such as dioxane) for cosolubilization of glycerol and fatty acid to overcome mass transfer limitations [1]. The monoglyceride fraction contains both monoglyceride positional isomers, 1-monoglyceride and 2-monoglyceride. Both isomers are similar in their surface activities but their melting properties are different [10]. The poor positional selectivity of the monoglyceride may be undesirable if production is designed for the development of the product intermediates. The 1-monoglycerides formed will not be optically pure (the carbon at the "2" position of the glycerol backbone is chiral), which can be a disadvantage if it is to be used as a food emulsifier since only one of the two stereoisomers (1-monoglycerides) is readily digestible by the enzymes of the human body.

1.3 Lipase Catalysis

1.3.1 Introduction

Enzymes are biological catalysts that exhibit activity under relatively mild conditions of pH and temperature. They are quite specific and stereo-selective in their actions. The properties, structure, kinetic mechanisms, and biochemistry of enzymes have been thoroughly reviewed [11,12]. Lipases, with the enzyme classification EC 3.1.1.1, are specific for the formation and cleavage of ester bonds containing fatty acids. In vivo, lipases are found near fat cells and are responsible for the hydrolysis of triglycerides, which is the first step in fatty acid β-oxidation, an energy-producing metabolic process. In addition, many organisms employ lipases to digest imbibed fats. Also, lipases are responsible for the synthesis of lipids needed for construction of cell walls and membranes. The reactions of lipase are reversible; fats can be formed through esterification or can be hydrolyzed

Lipases are easy to use because they operate under mild conditions with respect to temperature, pH, ionic strength, and so on. In addition, lipases do not require cofactors or coenzymes such as NADH. Furthermore, many species of lipase are readily available at relatively low costs. Lipases available commercially are derived from animal, plant, or microorganism sources.

Each lipase type has its own specific catalytic behavior. Each lipase has its own unique (but usually slight) preferences toward certain fatty acids as reactants, or substrates, based on fatty acid size and/or degree of unsaturation. Lipases can be divided into three major categories of specificity [13]:

(a) Those that are 1,3 positional specific. Such enzymes can cleave or form esters only at the outer hydroxyls of glycerol. The hydroxyl group at the middle or "2" position of glycerol is left intact. An example is the lipase found in the fungus *Rhizopus delemar*, which catalyzes the esterification to form 1-monoglycerides.

(b) Those that are specific for certain fatty acids. For example, lipase from fungus *Geotrichum candidum* possesses a strong specificity for esterifying/hydrolyzing unsaturated fatty acids with a carbon-carbon cis double bond at the 9-position. Another example is lipase from fungus *Aspergillus niger*, which is specific for short-chain fatty acids. Each lipase has its own unique (but usually slight) preferences toward certain fatty acids as reactants, or substrates, based on fatty acid size and/or degree of unsaturation.

(c) Those that have no strong specificities toward position or substrate. An example is the lipase from the bacteria Candida cylindracea which can completely cleave triglycerides into glycerol and fatty acids.

A good review on lipase types and their properties is available in the literature [14]. Catalytically, lipases act at or near interfaces [15]. This is not surprising when one considers the hydrophobicity of the substrates involved and the fact that lipases are employed in the synthesis of lipid bilayers. In terms of mechanism, lipases catalyze through the utilization of an acyl enzyme intermediate which is formed via a nucleophilic attack by lipase on an acyl-containing substrate (such as a fatty acid or ester) [16]. Nucleophiles such as alcohols and water then compete with the enzyme to bond with the acyl group.

1.3.2 Applications of Lipases

Industrial applications of lipases have been thoroughly reviewed [17,18]. Most of the applications involve lipase biocatalysis for production of specific products synthesized by hydrolysis or esterification, these products were briefly mentioned earlier. Lipase is added to many products to alter their composition and properties through its catalysis. For example, lipases are added to foods such as milk, cheese, and alcoholic beverages for fermentative purposes [18]. Also lipases are often added to detergents to assist in the breakdown of fats and greases [18]. Therefore, the demand for commercially available lipases is growing. The major problem in the application for lipases however, is the development of a proper reaction medium because lipases are most effective at the oil-water interface.

1.4 Types of Media for Lipase Catalyzed Reactions

1.4.1 Introduction

The selection of a medium for reactions using lipases is based on many factors. The primary one is solubilization of substrates such as fatty acids and glycerides, which requires hydrophobicity; but, to activate the reaction, the medium must have at least a small water content in order to hydrate the charged groups of the enzyme. Therefore, various types of biphasic and heterogeneous media have been employed. Most traditional biphasic media have been based in aqueous solutions. The major types, oil-in-water emulsions, micelles, lipid vesicles, and lipid bilayers, were compared in a comprehensive review [15] as reaction media for lipase. These media have been applied to carry out hydrolytic reactions, not esterifications, because the large water content limits the kinetic equilibrium of esterification. Such media also have other disadvantages when used as a host for lipase catalysis. For example, o/w emulsions, which are micron-sized dispersions of oil (commonly triglycerides) in aqueous solution solubilized by the action of emulsifiers, provide small interfacial area. This limits the quantity of sites available for biocatalysis. Furthermore, emulsions are thermodynamically unstable and require agitation. Lipid monolayers formed at air/water or oil/water interfaces, and vesicles which are spherical cavities encapsulated by lipid bilayers are the media that come the closest to mimicking in vivo lipase biocatalysis sites (lipid bilayers form biological cell walls or membranes), but their preparation can be difficult [15,19]. The solubilization of hydrophobic substrates is generally poor and they are very sensitive to the properties and behavior by small changes in substrate/product concentrations of media conditions Aqueous micelles are small dispersions of hydrophobic material in water stabilized by surfactants, such as bile salts, provide the best aqueous media for hydrolytic biocatalysis An aqueous micelle has a large interfacial area and preparation of aqueous micelles is relatively simple and reproducible Microemulsions (water-in oil type) have many advantages for hosting esterification reactions which will be discussed in the section 1 4 2.

1.4.2 Organic Media

Enzymatic catalysis in an organic medium has become an important research area in the past decade. It has many important advantages over aqueous medium. These advantages include

- low water content, desirable to shift thermodynamic equilibria away from hydrolysis
- solubilization of hydrophobic organic substrates,
- simpler separation and recovery of products and enzymes,
- limitation of microbial contamination
- changes in substrate specificity,
- enhanced thermal stability in organic media compared to water (water inactivates enzymes at high temperatures)

The pioneering research by Zak and Khbanov [20,21] has shown that many syntheses can be conducted in nonpolar organic solvents containing minimal amounts of water (less than 1 weight percent) through catalysis by suspensions of lyophilized enzyme powder A recent review by Dordick [22] discusses the preparation techniques, operating conditions, important considerations, and applications of enzyme suspensions in organic solvents Most of the research in this area has involved lipases that have catalyzed the following reactions fatty acid-sugar esterification [23], separation of racemic mixtures [7,24], lactone synthesis (formation of a component in musk oil fragrances) [25], formation of fatty acid-alcohol esters [21,26,27,28,29] glycol esterification [30,31,32] interesterification [33] and glycerolysis [34 35] of fats and oils A reasonably high conversion and enhanced enzyme stability can be achieved, however, the degree of success was strongly dependent on the water content [20] Although a small amount of water must be present to hydrate the enzyme's charged amino and carboxyl groups Any additional water promotes hydrolysis and lessens conversions Thus, an optimal water content exists, slight deviation from the optimal value lessens desirable levels of enzyme kinetics Lipases are reported to have half lives of 1.5 hours at 100°C, which represents extreme conditions for enzyme catalysis (most lipases are stable up to-40-50°C) [20] In addition the enzyme activity is very sensitive to additives like sugar and ions, which adversely affect the nature and quantity of the enzyme s bound water [25], and nonpolar organic solvents, which strip the enzyme s bound water [25] The effects of the water content in such media caused many disadvantages for esterification reactions by lipases For example controlling water concentration is important, but it is difficult to maintain the same water concentration, since water is often formed during the reaction (this is one reason why many authors in this field have examined trans and interesterification to form new esters since water is not a product formed). However, molecular sieves can be added at selected times and amounts to control the water content. The preparation of lyophilized lipase powders has a great influence on the reaction since impurities and ions affect the enzyme's bound water layer [25]. Another disadvantage is with enzyme recovery. Researchers in Klibanov's laboratory [36,37] showed that recovered enzyme powder recovered through filtration is quite active, but do not discuss the degree of recovery of enzyme activity. Large quantities of lipase can be lost to adsorption onto the filter membrane. This is one reason why immobilization of lipases has been investigated [28,38,39].

To improve enzyme-substrate contact, lipases have been made oil soluble by the addition of polyethylene glycol groups so that agitation would not be required. Results showed that modified lipases can catalyze a variety of reactions [28,38,39], but their reactivity is not greater than that of suspended crude lipases [39]. Lipase modified with dithiothreitol interacts with disulfide bridges between amino acid groups [40]. This modification improves operational stability but not thermal stability without loss of initial enzyme activity. In contrast, others found that lipase modified with glutaraldehyde enhanced catalytic stability, but significantly reduced enzyme activity [41]. Klibanov suggests that the slight gain in enzyme reactivity is not worth the extreme effort required for the tedious and imprecise derivation techniques [21]. Moreover, enzyme recovery is hindered by its solubility in the media. Researchers in Japan have derivitized lipases to contain metal so that lipases can be recovered from the media by applying a magnetic field [42].

Lipases immobilized on matrices have many applications. The major advantage of immobilization is simplified recovery of enzyme activity. Also, immobilized enzymes can be evenly dispersed in organic solvents [25]. A recent review [43] discusses types and methods of immobilization, choices of immobilization, and process parameters. The methods of immobilization of lipases include physical adsorption onto solid porous supports, entrapment into gels or resins, microencapsulation, and covalent cross linking of lipases with each other and/or to a support. Support matrices for lipase immobilization include porous glass, silica, clays and ciliates, and dextrans such as Sephadex.

Lipase adsorbed onto matrices have been used in organic solvents in a variety of applications, including oil modification by interesterification [13,44,45] or transesterification [45,46], alcoholysis by glycerol [45], production of fatty acid-alcohol esters [8,40,41,47,48], separation of racemic alcohols [49], and hydrolysis of penta-esters of glucose to produce other glucose esters [50]. Immobilized lipase catalysis produced high yields of esterification [8,13,41,51] and excellent retention of enzyme stability [48,51]. This allowed the design and use of packed bed reactors of immobilized lipase [13,43,52,53]. But rates of conversion were often slow due to activity loss during the immobilization process [43] or mass transfer limitations [54]. Overall, these types of media for lipase have potential for future large-scale use (depending on cost analyses), especially with respect to recovery of enzyme activity, but their sensitivity to water content and enzyme impurities [53,55] can cause reproducibility problems. Another drawback is the relatively low rate of product formation from these lipase preparations. Although immobilized lipases have generally shown good retention of enzyme activity, lipase stability in scaled-up reactors for long term use must be carefully approached since enzymes can slowly degrade with time, temperature, agitation, and the presence of water [43].

Esterification and related reactions have been investigated with immobilized or "free" lipases in the presence of substrates [56-59]. The major advantage of such operations is the simplification of downstream separation processing. Also, the absence of solvents will improve operational safety and promote higher substrate concentrations. But in the absence of organic solvents, reaction media can be quite viscous, due to the innate properties of such common substrates as triglyceride oils and glycerol. These are usually batch processes. Scale-up will be hindered by additional pumping and heating costs. The development of continuous reactor design such as a packed bed reactors will be difficult due to the high pressure drops imposed by viscous fluids [52]. Omar and his group [56] esterified glycerol and oleic acid in the presence of crude lyophilized lipase from *Humicola lanuginosa* at room temperature with the excess of oleic acid. They received a 75% conversion after three days. But, the product distribution was mixed, with the major product being monooleins. When thay tried esterification without organic solvents between oleic acid and monohydroxyl alcohols, the conversion was 50% [56]. Ergan's research group has successfully produced an esterification reaction which contains almost entirely triolein in a couple day's time using oleic acid and glycerol in the absence of organic solvents [57 58] Water removal by molecular sieves was the key towards triglyceride formation. The addition of the sieve at the latter stages of the reaction is important to promote esterification of mono and diglycerides [57] Another very interesting result was achieved with a 1,3 positional specific lipase catalyzed esterification. This was achieved by a rapid, noncatalyzed acyl migration, where 1,3 diglycerides isomerized to 1,2 diglycerides this isomerization was promoted by a high oleic acid content [59] McNeil et al [34 35] has used glycerolysis of oils and fats without organic solvents by a variety of free, lyophilized lipases to produce a product distribution containing 80-90% monoglycerides within five days by monitoring temperature. A high temperature was first applied to produce rapid esterification, then the temperature was reduced and held constant for a few days. The lowering of the temperature promoted crystallization of the monoglycerides, which prevented its further esterification toward di- and triglycerides and promoted favorable equilibrium for further glycerolysis [35] Here the problem was the recovery of lipase [35].

A unique membrane bioreactor for continuous lipase catalyzed esterification between glycerol and oleic acid has been developed [60,61], where a stream of oleic acid comes in contact with a polar stream containing glycerol lipase, and a small quantity of water through a hydrophobic membrane [60,61] The lipase adsorbed onto the membrane allowed penetration of the oleic acid and glyceride products only due to the hydrophobicity of the membrane. Here the maximum esterification of

80% was obtained with equimolar concentrations of mono- and diglyceride. The polar stream occasionally was stripped of some of its water content by molecular sieves, and some enzyme activity was lost from the bioreactor in 3-4 weaks [61]. As a limitation, the maximum conversion and the product distribution could not be improved upon by addition of lipase or by water removal [60]. Also, with time lipases slowly desorbed from the membrane [60]. Potential problems with scale-up of all membrane bioreactors are related to shear tolerances of membrane, large residence times needed for biocatalysis, and difficult maintenance procedures [61].

Another reaction media having a significant potential are w/o microemulsions, which are colloidal aqueous dispersions in organic solvents. This is the medium chosen here for hosting lipase catalyzed esterification reactions. The properties, advantages and applications of microemulsions for lipase-catalyzed esterification are discussed as follows.

1.5 Microemulsions

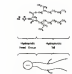
1.5.1 Introduction, Description, and Applications

It is well known that oil and water do not mix. The origin of this phenomenon is the hydrogen bonds of water. The free energy that might be gained from solvating some nonpolar molecules (from weak Van der Waals forces) is small compared to the free energy that would be lost from breaking a hydrogen bond [63]. Thus when oil and water are mixed, an inhomogeneous, thermodynamically unstable, emulsion forms that separates into two components over time.

It is, however possible to mix polar liquids like water, glycerol, or acetonitrile with nonpolar hydrocarbons such as *n*-alkanes to form thermodynamically stable dispersions with the help of suitable surfactants. This phenomenon was reported by Hoar and Schulman [64] who proposed the "oleopathic hydro-micelle" model on which today's reverse micelles is based. Although Hoar and Schulman [64] were working with the surfactant stabilized water droplets, single phase mixture of surfactant, water, and oil may not always contain discrete water droplets.

Microemulsions may be defined as thermodynamically stable isotropic dispersions of two relatively immiscible liquids, consisting of micro-domains of one or both liquids stabilized by an interfacial film of surface active molecules called surfactants [65]. Surfactants possess an amphiphilic structure, meaning they consist of both hydrophilic and hydrophobic parts in their molecular structure, which makes them concentrate at oil/water interfaces. Surfactants may be anionic, cationic, or nonionic depending on whether the hydrophilic part is negatively charged, positively charged, or neutral. A commonly used anionic surfactant, called dioctyl sodium sulfosuccinate (Aerosol-OT or AOT) with its hydrophilic anionic sulfonate "head" group and the two hydrophobic "tail" groups is shown in Figure 1.2. Figure 1.2 also shows a water-in-oil microemulsion droplet stabilized by AOT.

There are three easily identified types of microemulsion. An oil-in water (o/w) microemulsion consists of a small amount of oil dispersed in a continuous water phase in the presence of surfactant. In such a system, head groups are directed out towards the water and non-polar tails are pointing inward. The solubilized oil



Dioctyl sodium sulfosuccinate (Aerosol OT or AOT)

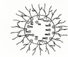


Figure 1.2 AOT and schematic representation of an AOT w/o microemulsion droplet.

is dispersed into the interior of the droplet. A water-in-oil (w/o) microemulsion is the dispersion of a small amount of water in a continuous oil phase. In this case head groups and any counter ions are localized in the interior of the aggregate, the water is solubilized in the core of the microemulsion droplet and the surfactant exhibits negative curvature. In the third type, when volumes of oil, surfactant, and water are similar, the mixture shows a bicontinuous system which exhibits fluctuating curvature (both positive and negative). There is no dispersion of one phase into another phase in the bicontinuous system, each phase may be regarded as occupying opposite sides of continuous, interconnecting surfactant-coated channels [66]. Such bicontinuous microemulsions often possess high viscosities as compared to o/w or w/o microemulsions because of the extended structures and, like an o/w microemulsion, it shows high conductivity due to the presence of water channels. Figure 1.3 shows various structure formations in surfactant solutions. The hydrodynamic radii of the droplets are within a very narrow range, so droplet sizes are reasonably monodispersed. It can be shown that the droplet radii are a function of the molar ratio of water to surfactant (w_0). This parameter w_0 signifies both composition and droplet size.

The main driving force for the formation of a w/o microemulsion arises from the reduction of the interfacial tension (surface energy per unit area), resulting in a reduction of the Gibbs free energy of the system.

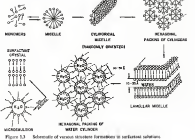

MONOMERS
MICELLE
CYLINDRICAL MICELLE (RANDOMLY ORIENTED)
HEXAGONAL PACKING OF CYLINDERS
SURFACTANT CRYSTAL
H_2O
10-35 Å
10-35 Å
WATER
OIL ALCOHOL
LAMELLAR MICELLE
MICROEMULSION
HEXAGONAL PACKING OF WATER CYLINDER

Figure 1.3 Schematic of various structure formations in surfactant solutions

The Gibbs free energy equation is shown below

$$\Delta G_f = \gamma \Delta A - T \Delta S$$

where G_f is the Gibbs free energy (in Joules). Δ represents a small change in the subsequent parameter, T is the absolute temperature in degrees Kelvin, S is the entropy (J K^{-1}), A is the surface area of the interface in m^2, and γ is the interfacial tension (N m^{-1})

As compared to the area of phase separated system, there is an increase in the area (A) of several orders on mixing oil, water, and surfactant to form a microemulsion. In order to compensate for this effect, there must be a concomitant reduction of the same order of magnitude in the interfacial tension. This decrease in the interfacial tension is achieved by the packing of surfactant molecules at the interface between the polar and non-polar phases, thus preventing oil and water from coming into direct contact [67]. However, change in the entropy also affects the free energy term. While, the dispersion of one liquid, in the form of very small droplets, into the other will increase the entropy of the system, but the association of surfactant molecules at the interface can significantly reduce the entropy. This unfavorable lowering of entropy is thought to be minimized by the thermal fluctuation of the interface which reduces ordering in the surfactant monolayer and by surfactant motion over the interface and surfactant exchange between the interface and the continuous oil phase. The net change in entropy associated with microemulsion formation is generally considered to be positive and therefore contributes to the lowering of the free energy term.

1.5.2 The Effect of Surfactant Structure

The manner in which surfactants pack at an interface is dependent on the molecular geometry. As a consequence, this affects the type of organized assembly formed under fixed conditions of temperature and pressure. In an attempt to quantify this effect Mitchell and Ninham [68] introduced the concept of a surfactant packing parameter($\mathbf{Sp}$) such that

$$\mathbf{Sp = V/AxL}$$

where $\mathbf{V}$ is the volume of the surfactant's hydrocarbon tail, $\mathbf{L}$ is the length of the hydrocarbon tail, and $\mathbf{A}$ is the headgroup area at the oil-water interface, which is determined by optimizing the opposing forces of headgroup repulsion and minimizing water-hydrocarbon contact. The Mitchell and Ninham approach has shown that for structures having positive curvature, such as spherical and cylindrical micelles and o/w microemulsion, $\mathbf{Sp < 1}$, laminar and bicontinuous structures where there is very little net interfacial curvature, give $\mathbf{Sp = 1}$, and structures possessing negative interfacial curvature, such as reverse micelles and w/o microemulsions, give values of $\mathbf{Sp > 1}$

Using the pocking parameter analysis, one can see why twin-chain surfactants like AOT show more tendency to negative curvature than single-chain surfactants like CTAB since having extra tail has a much greater effect on $\mathbf{V}$ than on $\mathbf{A}$. The Mitchell and Ninham approach also goes some way to explain the co-surfactant effect in that, by adding a medium chain-length alcohol, $\mathbf{V}$ is increased relative to $\mathbf{A}$, thereby promoting formation of w/o microemulsions. This effect is frequently observed for CTAB in linear and branched alkanes [68].

Interestingly, Martin and Magid [69] observed that AOT is capable of changing its Sp by simply altering the relative orientations of the two hydrocarbon tails. When this is done the Sp of the trans-isomer is lower than that of the cis isomer. At high R-values where there is more water, thus reduced negative curvature the proportion of trans-isomer is increased. This makes the wedge-shaped surfactants less broad at the base, thus encouraging more efficient packing in a near planar arrangement. This more efficient packing is thought to be a major factor contributing to the stability and high solubilization capacity of AOT microemulsions.

The phase diagram of water/AOT/organic solvent media has been well documented [70]. Temperature is also an important factor in influencing microemulsion properties and phase composition. Also, in many cases the role of the surfactant needs to be supported by a cosurfactant, such as short or medium-chained alcohols (e.g. 1-pentanol), in order for water to be solubilized.

Microemulsions have many unusual, interesting, and important properties. They are thermodynamically stable and do not require agitation for their formation; the nano size of the dispersed droplets of 100 - 1000 Å gives a large polar/nonpolar interfacial area [71]; the size or diameter of aqueous droplets in w/o microemulsions is proportional to the molar ratio of dispersed phase-to-surfactant present, the dispersed droplets are spherical and monodispersed, and microemulsions are dynamic in nature so the droplets are constantly colliding with each other, leading them to coalesce and breaking apart on a time scale of milliseconds [72,73,74]. In addition, the water solubilized in w/o microemulsions has different properties compared to bulk water. These are primarily due to the disruption of hydrogen bonding between water molecules by the "head" group of the surfactant and to the water to-surfactant ratio, w_o. For AOT-containing reverse micelles at w_o values less than ca. 4 the water is strongly bound to the surfactant's sulphonate group and sodium counterion, as w_o is increased toward ca. 10, the water added is still bound to the "head" groups, but not as strongly, so it is only for w_o values of 10 or less that water has properties, such as increased microviscosity, lessened polarity, and reduced freezing point, significantly different than that of bulk water. These altered properties, which have been compared to strongly bound water near biological membranes, and the conditions at which they are altered are discussed in various reviews [71,75,76]. Furthermore, at low values of w_o, the actual pH of the water "pools" may be different from the apparent value, the difference is lessened when a buffer salt is employed at high concentrations and the pH value near its pKa [77]. As w_o values are increased, the added water is not strongly bound to the interface and this "free" water has properties more like those of bulk water.

Microemulsion droplet sizes and their behavior are very sensitive to the presence of additives. For example, the presence of high ionic strength aqueous pseudophase decreases micellar sizes and the micellar collisions become more like "hard" spheres [78]. The presence of long-chained cosurfactants causes the same behavior, while short-chained cosurfactants reduce interfacial curvature and promote more attractive collisions [79]. The solubilization of large molecules, such as proteins, causes great changes in the structure of the microemulsion. When proteins are present in microemulsions, there exists a bimodal distribution of sizes: one for protein-containing, or "filled" micelles and one for "empty" micelles [80,81]. This phenomenon will be discussed in more detail below.

Microemulsions have many applications in biotechnology. Most of these have been listed and discussed in a recent review [82]. One of the most interesting and important applications is for the extraction of proteins and amino acids. This process, investigated by many research groups worldwide [83,84,85,86,87], involves the selective migration of proteins or amino acids from an aqueous phase into a w/o microemulsion, with the major driving force being an electrostatic potential field between the net charge of the protein and the charge of the "head" group of the surfactant. "Back extraction" (done by increasing the ionic strength of the aqueous phase) into a fresh aqueous phase can then be used to reobtain the proteins. Recent thermodynamic models illustrate the importance of electrostatic interactions between all ionic species with respect to protein uptake in microemulsion droplets; model generated data correlate well with extraction data [88,89].

Specific areas of applications include enhanced oil recovery [90,91], oil-clean up [92], catalysis [93], lubrication [90,94], preparation of inks and paints [95], photochemistry [74], separation and recovery of ions [96,97], and polymerization [98]. Additional applications of microemulsions also include drug delivery systems [99] and models for mimicking in vivo systems. Microemulsions containing solubilized nucleic acids may accurately model viruses and phages [100], while microemulsions containing proteins and enzymes may be studied as representations of membrane proteins [101,102]

1.5.3 Enzymatic Reactions in w/o Microemulsions

Enzymatic reactions in w/o microemulsions have received a great deal of attention in the last ten years with many comprehensive reviews being written on this topic [74 75,103,104 105] There are many advantages to using biocatalytic operations in w/o microemulsions. These includes substrate solubilization and lower water content Of the various biphasic media in existence, w/o microemulsions have the largest interfacial area, thus potentially yield impressive enzyme-substrate interaction. In addition, since w/o microemulsions collide and exchange materials within microseconds or even faster, mass transfer limitations are not significant. Another important advantage is the ease of preparation, no tedious immobilization or emulsification techniques are required. Methods of preparation, which mainly involve the introduction of enzyme to the media by the injection of an aqueous enzyme buffer solution, solubilization of dry lyophilized enzyme powder in micellar media, or micellar extraction from an aqueous phase, are described in detail elsewhere [104]. Also, for certain reactions kinetic analysis can be monitored with ultraviolet spectroscopy for certain reactions. Furthermore, kinetic results obtained in microemulsion systems have high degrees of reproductibility

There are however major disadvantages in reactor design and process scale up Operation in w/o microemulsions is inherently a batch process For continuous process, the compartmentalization of enzyme-containing w/o microemulsions in hollow microfibers from a bulk organic solvent was proposed [106]. This would be particularly advantageous for reactions that produce hydrophobic products, because the product can easily be recovered by recirculating the bulk organic fluid. However, most species interact to some extent with the micellar interface. Thus, in many cases, after the reaction is complete the micellar structure would have to be broken. Also, results from this type of reactor have been discouraging [106], perhaps due to the mass transfer limitation imposed by the microfiber membrane. Another disadvantage involves product recovery from a batch process. The difficulties are caused primarily by the presence of the surfactant and a practical scheme for recovery of materials has yet to be developed. Despite these problems, the recovery of enzyme activity is feasible through micellar back-extraction

In terms of reverse micellar enzymology, also called back-extraction there are some important and controversial findings. The rate of production, symbolized by the "turnover number," k_{cat}, are enhanced for chymotrypsin [103,107,108]. A recent thermodynamic model using AOT as the model surfactant predicts the possibility of enhanced activity, due to the electrostatic repulsion of anionic substrates from the interface [109,110]. In general, in work conducted with hydrophilic enzymes and substrates, the values of k_{cat} found in w/o microemulsions have been similar to values found in aqueous or other media; cases of "superactivity" display only modest gains [74]. However, in the analysis of Michaelis-Menten enzyme kinetics applied to w/o microemulsions [103-105], in some cases differences in the value of the Michaelis

Constant, K_M, representative of the stability of the enzyme-substrate intermediate were discovered from the same reaction in aqueous media [74,105]. The larger values of K_M for reactions in w/o microemulsions could be attributed to more stable enzyme substrate complexes. Accurate kinetic modelling for reactions in w/o microemulsions has not been achieved.

A third interesting result involves the relationship between the rate of production, k_{cat}, and w_o, the water surfactant ratio, which is proportional to micellar size. In general, the curve of k_{cat} versus w_o is bell shaped, indicating that an optimal w_o value exists [104,105]. The optimal value of w_o often corresponds to a micellar size in the absence of enzyme which is nearly identical to the volume size of the enzyme itself [111]. This observation has brought about many possible theories. Recent modelling attempts, by thermodynamics/electrostatics [109], diffusion [111], and size distribution of w/o microemulsions [112] have all duplicated the bell-shaped dependence. A recent model [113], based on population balance techniques like that in one of the electrostatic models [109] attributes the optimum value of w_o to enhanced substrate concentration in the vicinity of the enzyme. The diffusion model [111] is the only model of these to relate the bell-shaped dependence to the size of the biocatalyst, although, as written their model is difficult to fully comprehend. Experimental data suggests that reduced aqueous polarity also may be a factor [102]. Another reason attributed to the bell-shaped dependence, as well as the case of "superactivity," is enhanced conformational changes of enzymes, but this topic is controversial. It is generally true that enzymes do not change structure at all or only slightly in w/o microemulsions [75,76], except in the absence of substrates [114] or needed ions [114]. Although one research group has found evidence of changed substrate specificity upon micellization for liver alcohol dehydrogenase [116]. Another kinetic difference was noted by Walde et al. [115], who observed that the rate-limiting substep of hydrolysis for the enzyme trypsin was different in w/o microemulsions from that in aqueous media. Enzyme stability is also an important factor. A recent review by Luisi and Magid [75] reports that, in general, enzyme stability in w/o microemulsions is similar to that found in aqueous media, and is greatly enhanced at low values of w_o. Unless substrates are present, a rapid deactivation of enzymes in w/o microemulsions is noted.

Determining the location of enzymes in w/o microemulsions is an interesting problem. It is suggested that more hydrophilic enzymes such as chymotrypsin solubilize entirely in the micellar water "pool," while more hydrophobic and-surface-active enzymes such as lipases solubilize at or near the micellar interface [103]. This phenomenon can have an extremely important role in the observed kinetics, as a recent model indicates [117]. Determination of enzyme solubilization is experimentally very difficult due to the dynamic nature of the media. Speculation on enzyme solubilization has been based upon indirect methods. It has been only recently that the most direct type of evidence, measurement of differences between "filled" and "unfilled" w/o microemulsions, has been obtained [103], but this evidence is controversial. The method of using Small Angle Neutron Scattering contains many assumptions, including that a w/o microemulsions can contain at most only one protein molecule but this assumption has been questioned by recent findings [118] The double dye ultracentrifugation technique of Zampieri et al. [81] yields reliable results only for very low water content, but does have potential utility Other techniques that measure micellar size alone, such as light scattering and fluorescence, require a large overall concentration of protein to be present; this can be experimentally difficult to achieve as well as potentially being expensive Also, complications such as enzyme-enzyme interactions at high aqueous concentrations may make interpretation of results difficult, although, two studies employing such techniques have indicated that Cytochrome C [118] and Myelin Basic Protein [119] do strongly interact with the interface.

Microemulsion technology is important for the exploration of alternate types of media, especially those that employ natural or "edible" surfactants, since application of these would meet with the approval of the food industries, for example, where potential toxicity is an important consideration. Other areas needing continued research include exploration of other important applications like, the-scale-up of w/o microemulsion enzymatic reactions, and the modelling of the incorporation and solubilization of substrates proteins, and biomolecules to achieve a better understanding of the forces and interaction involved.

1.6 Lipase Catalyzed Reactions in Microemulsions

The w/o microemulsions have many advantages for hosting the esterification reactions of lipases. These include low water content, ease of preparation, large quantities of polar/nonpolar interfacial area, and no mass transfer limitations because of the rapid rate of droplets collisions and exchange of materials. Two other advantages exist for w/o microemulsions over its competitors; both are related to the encapsulation of polar materials. First, reactions in w/o microemulsions do not appear to be quite as sensitive to water contents compared to other organic solvent media due to their compartmentalization inside w/o microemulsions and larger water contents can be tolerated. Second, by encapsulation, large quantities of hydrophilic and viscous alcohols and polyols can be solubilized in organic media without the need of elevated temperatures or agitation.

Hydrolysis is involved in most of the investigative work in microemulsions. Substrates employed for hydrolysis include triglyceride oils [120,121,122,123], p-nitrophenyl alkanoates which reactions which can be monitored in situ by Ultraviolet Spectroscopy [124,125], and fatty acid methyl esters [126]. Various lipase types also have been investigated. Han and Rhee, for example, have studied hydrolysis of olive oil by lipase from *Candida cylindracea* using AOT-containing w/o microemulsions [120,127,128]. Their results illustrate that under most conditions enzyme kinetics behave similar to those encountered in aqueous media with respect to pH and Michaelis-Menton model [120,126]. It is the consensus of all researchers who have investigated lipase hydrolysis in AOT micellar media [121,124,129,130,131], that a bell-shaped relation between k_{cat} and w_o existed for various lipases. Important findings for hydrolytic reactions include those of Fletcher et al. [124] who correlated substrate partitioning with observed kinetic trends. While some investigators have examined enzyme activity retention of lipases in w/o microemulsions, their results are mixed Fletcher et al. [124] report lipase from *Candida viscosum* retains 60-85% of its origional activity after one week [124]. Han and Rhee [120] report rapid deactivation for lipase from *C. cylindracea* in large w/o microemulsions, while in micelles at small values of w_o, eighty percent of the original activity is retained. In the case of pancreatic lipase Malakhova et al. [121] also report significant enzyme deactivation. Even though the reduction of w_o greatly improved enzyme stability [120 124], the degree of deactivation in all cases was at an industrially unacceptable level As Han and Rhee suggest [120], the presence of substrate needs to be included in the analysis of enzyme stability, in the cases reported here, it was not. In terms of reaction media investigated, AOT-based media were shown to be superior in terms of rate and extent of hydrolysis compared to media containing other surfactants [122,124,125,129,132] In addition, it was found that certain nonionic surfactants underwent undesirable side reactions [122]

Han and Rhee suggest that microemulsion systems is the assay medium of choice for lipase since it promoted rapid catalysis, repeatable kinetic results, and allows the employment of various substrate types [132]. Recently, Peter Walde and his associates in Zurich have enhanced the potential application of using w/o microemulsions for assays by developing two different techniques for in situ detection of the progress of the reaction. The first involves the inclusion of the pH indicator phenyl red into the aqueous pseudophase [134] This reagent loses its red color as more fatty acid is formed. So the decrease in absorbance is linearly proportional to the overall fatty acid concentration [134]. The second is with Fourier Transform Infrared Spectroscopy, where changes in the ester and free fatty acid carbonyl stretching region are proportional to kinetic changes [135]. In terms of large-scale application, a research group from Sweden [129] is investigating the use of w/o microemulsion media to synthesize 2-monoglycerides, important food emulsifiers, from triglyceride oils with 1,3 positional specific lipases. They found, however, that if the reaction was allowed to continue for too long, 2 monoglycerides isomerized to 1-monoglycerides that were readily hydrolyzed [129]. They also found that AOT systems yielded larger amounts of monoglyceride product and lesser amounts of glycerol, formed after the isomerization, than nonionic surfactant-based media [122]. In order to employ lipase-catalyzed hydrolysis to produce 2-monoglycerides or "free" fatty acids, larger concentrations of triglyceride substrates will have to be employed and investigated.

The one most frequently researched is the esterification of fatty acids and normal alkane alcohols [130,136,137,138]. In most cases, conversions were 70-90%, but depended upon the nature of the substrates. For example, employing lipase from *C. viscosum* in AOT media produced high conversions when octanol was the alcohol substrate but low conversions when ethanol was substituted [138]. A relationship between the chain length of the alcohol and the initial rate of esterification was also obtained by Morita et al. [130] for catalysis by lipase from *Rhizopus delemar* in phosphatidyl choline w/o microemulsions. Another interesting relationship noted in this reaction was that the order of adding substrate and aqueous enzyme solution to the surfactant-organic solvents media had a large impact on the rate of esterification [137]. The research group of Bello et. al. also obtained a high degree of esterification for this reaction using the nonionic surfactant Tween, when oleic acid and alcohol were substrates [136,139]. Alcohol was the necessary cosurfactant for formation of optically clear micellar media. However, the reaction time for their media to achieve a large conversion was over two weeks, a much longer time than that reported by others for lipase biocatalysis in w/o microemulsions [136].

The group of John et. al. [140] have investigated triacetin/tributyrin transesterifications in AOT-hexane media. They found that transesterification occurred only to an equal or lesser extent than triglyceride hydrolysis [140]. Also, they determined that transesterification was obtained between triolein and triglycerides containing short chained fatty acid groups employing the nonionic surfactant Tween and a cosurfactant to form w/o microemulsions in the absence of additional organic solvent [136,139]. Their results indicated a transesterification of 40%, in weeks [136,139]. Furthermore, the authors mentioned that hydrolysis occurred significantly, but did not quantify the extent [136]. The research group of Holmberg and Osterberg [141, 142] have investigated the reactions of interesterification between palm oil and stearic acid, for synthesis of cocoa butter substitute using microemulsion systems. They found that micellization promoted lipase biocatalysis of 20-30% stearic acid interesterification within one to two days [141]. The extent of interesterification was similar to that catalyzed by celite-adsorbed lipase but micellization promoted faster rates of reaction [141]. Similarly, they found that micellar media employing AOT or nonionic surfactants promoted biocatalysis for the reaction, but that the nonionic type of system also promoted a side-reaction between substrate and surfactant [141]

The same researchers [143] recently employed the surfactant AOT in isooctane with a 1,3 positional specific lipase to obtain monoglycerides from reacting glycerol and palm oil [143] Their overall yields were somewhat low, less than two moles monoglyceride produced per mole triglyceride present initially, and the extent of hydrolysis, as noted by the fatty acid content, was nearly equal to that of the extent of glycerolysis [143] With a positional specific lipase, hydrolysis would be desirable since 2-monoglycerides would be potential products, but significant isomerization to 1-monoglycerides was detected [143]. The large pool of 1-monoglycerides formed from the isomerization of hydrolysis products and from glycerolysis was hydrolyzed to a significant extent, probably due to thermodynamic equilibrium limitations [143]

Fletcher and Robinson [138,144] demonstrated the feasibility of the esterification reaction between glycerol and fatty acid in w/o microemulsions They employed the non-positional specific biocatalyst lipase from *C. viscosum* in a medium composed of AOT and isooctane with substrate and a small amount of water [144] They reported conversions as high as 90%, with a product composed mostly of-mono-and diglycerides at relatively similar amounts [138] Thus, the extent of esterification was not great enough for the synthesis of triglycerides, as it was for immobilized lipases when water removal was adequate [58] They discovered that the final "equilibrium" distribution was quite dependent on the water content [144] But the amount of fatty acid in oil phase was very low (<6% by weight) which limits process development using this route.

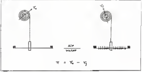
Morita et al [130] reported that they achieved glyceride synthesis in phosphatidylcholine w/o microemulsions employing lipase from *R. delemar*, which is positional specific. They state that they produced mono- and diglycerides [130] They illustrate results from the esterification of 1,2-diglycerides and fatty acid that show about 75% conversion of the diglyceride, of which one half to two thirds of the product were triglycerides and the other third monoglycerides from hydrolysis, the reaction time was four hours [130]

The investigations of Morita et al [130] and Fletcher et al. [138, 144] left many questions unanswered, including to what extent can esterification be achieved and which operating parameters would be important. They did not identify as to what physical and/or kinetic factors dictate the kinetics All the w/o microemulsion systems reported for both hydrolysis or esterification are based on non-reacting surfactants, like AOT, CTAB, and Tween. The solubilization of oil-soluble substrate in the oil phase is at present very low (<6% by weight for esterification), which limits potential process development. Finally, the separation cost of products would be very high. These problems and disadvantages point to the need for research on a new microemulsion system. In our study, an attempt has been made to develop w/o microemulsion using fatty acid as a substrate, plus sodium salt of same fatty acid and tertiary butanol

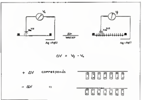
1.7 Monolayers

A typical surface active molecule consists of a polar group and a nonpolar group (hydrocarbon chain). This separation of hydrophilic and hydrophobic parts of the molecule causes such a molecule to adsorb at the air-water or oil-water interfaces, which causes decrease in surface or interfacial tension. The surface active molecules, when spread at the air-water interface, will be insoluble in the subsolution if their hydrocarbon chains are sufficiently long (>16 carbon atoms). The presence of a polar and a nonpolar group in the molecule allow it to orient at the interface and form an insoluble monolayer. Monolayers represent a two dimensional state of matter because their height is negligible compared to their length and width of the monolayer. The surface properties of these monolayers can be measured by various techniques. The surface pressure, surface potential, and surface viscosity are the most commonly measured parameters to characterize monolayers. Surface pressure can be measured with a Wilhelmy plate and a Langmuir-Adam horizontal film balance. In the Wilhelmy plate method, a thin plate, usually of glass, quartz, or platinum, is suspended in the liquid from a torsion balance (Figure 1.4). The surface of the plate is roughened to have a zero contact angle between the liquid and the plate. A monolayer of a surface-active material can be spread by dissolving it in an organic solvent.

The area per molecule can be calculated by dividing the total area of the monolayer by the total number of surface active molecules. The limiting area of a surface-active molecule is the area per molecule of the monolayer at its collapse



a. Measurement of Surface Pressure (π)



b Measurement of Surface Potential (ΔV)

Figure 1.4 Schematic of surface pressure and surface potential measurement methods.

pressure. Normally, results are shown by a surface pressure-area (π-A) plot.

The surface pressure (π) of a monolayer is defined as

$$\pi = \gamma_o - \gamma_r$$

where γ_o is the interfacial tension of the subsolution without the monolayer, and γ_r is the tension in the presence of the monolayer

The surface potential of a monolayer depends primarily on the characteristics of the polar groups in the surface active molecules. According to Shah and Schulman [145] it is the most important parameter for studying ionic interactions in monolayers The two major techniques used for measuring the surface potentials of monolayers are the radioactive electrode method and the vibrating plate method.

In the radioactive electrode method, one terminal of an electrometer is connected to an electroplated Ag-AgCl electrode which is submerged in the subsolution and a second terminal is connected to a metal rod The tip of the metal rod is covered with a radioactive material (α-emitter) positioned 1-4 mm above the surface The α-particles from the radioactive material ionize the air gap, reducing the electrical resistance between the electrode and the interface to such a low degree that the internal resistance of the electrometer is considerably greater than that of the air gap (Figure 1 4) This results in the completion of the circuit. This method is very convenient for studies at the air-water interface, but not at the oil-water interface because the range of α-particles in the oil is very small compared to that in air

The surface potential (ΔV) of a monolayer is defined as

$$\Delta V = V_1 - V_o$$

Where V_f and V_o is the interfacial potential in the presence and absence of the monolayers , respectively Surface potential data are exhibited by ΔV-A curves analogous to the π-A curves for monolayers.

1.7.1 Enzymatic Reactions in Monolayers

The enzymatic hydrolysis of lecithin monolayers by snake venoms were reported by Hughes by measuring changes in the surface potential [146]. This approach has been used by numerous researchers in the kinetic study of hydrolysis of monolayers Shah and Schulman [145] reported the influence of divalent cations, different buffers, the presence of other lipids, and unsaturation on the hydrolysis of dipalmitoyl, egg, soybean, and dioleyol lecithin monolayers by the venom of Naja naja. It was shown that a lecithin monolayer does not hydrolyze when it was compressed above a critical surface pressure The critical surface pressure required to block the penetration of the enzyme into the monolayer increased with molecular area (i.e., dioleyol lecithin > soybean lecithin > egg lecithin > dipalmitoyl lecithin) The rate of hydrolysis was measured by the decrease in the surface potential It was observed that hydrolysis does not occur above a certain critical surface pressure. Hydrolysis depends on the intermolecular spacing among the molecules and the charge on the molecule. Shah and Schulman have shown that when 15 mole % of anionic, cationic, or neutral spacer molecules were introduced into egg lecithin monolayers, the rate of hydrolysis is changed. The rate of hydrolysis increases in the presence of eicosanyl trimethylammonium, decreases in the presence of dicetyl phosphate or dipalmitin, and remains unchanged in the presence of cholesterol. This shows the importance of surface charge and state of the monolayer for enzymatic reaction.

The presence of a net negative charge is a prerequisite for the hydrolysis of ^{32}P-labeled yeast lecithin monolayers by *Penicillium notatum* phospholipase, as measured by the loss of radioactivity [147]. The surface pressure, surface potential, and radioactivity measurements indicated that, in the absence of dicetyl phosphate, lecithin monolayers are not hydrolyzed by phospholipase B at high surface pressures, whereas in the presence of anionic amphipathic molecules, the hydrolysis does occur [148]. The enzyme can penetrate deeply enough to hydrolyze the lecithin monolayer at low surface pressure, whereas at high surface pressure, a negatively charged surface is required to pull the enzyme into the monolayer to hydrolyze it [148]. Alexander and Rideal [149] measured surface pressure and surface potential during the hydrolysis of long chain esters in monolayers on both alkaline and acid subsolutions. They found that the reaction velocity is very sensitive to changes in the molecular orientation of the long-chain esters in the monolayers.

There is no report in the literature of the enzymatic synthesis in the monolayer. Monolayers of fatty acid can be made on the glycerol and water subsolution. Lipase solution can be injected under the compressed monolayer to initiate the reaction between glycerol and fatty acid (Figure 1.5). Esterification reactions in monolayers

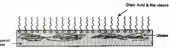


Figure 1.5 Schematic representation of esterification reactions in monolayers.

were compared with the esterification in microemulsions. Partitioning of products in the oil phase is possible in the case of a water in oil microemulsions, but not in the case of monolayers at air water interface. The orientation of substrate or product at the air-water interface and its packing can influence the esterification in monolayers. Also, the acyl migration of fatty acid in monoglyceride and diglyceride has been reported. This may be due to the same kind of favorable orientation during its thermal motion which triggers acyl migration.

1.8 Reactions in Foams

Foam is an aggregate of air bubbles separated by thin liquid films. These liquid films contain two air-water interfaces. Foam also is a thermodynamically unstable system. Interestingly, foam as a compound exhibits a much higher viscosity than its constituents, namely the aqueous solution of surfactant and the gas phase. The stability of foam depends on the stability of its bubbles. The stability of a bubble is related to the rate of drainage and hence the rate of thinning of the film. It has been suggested that the surface viscosity of the adsorbed surfactant layer influences the rate of drainage. High surface viscosity retards the drainage, hence it improves the film stability. The surface viscosity of the monolayer depends on molecular packing and molecular interactions.

Foam consists of air-water interface which can be stabilized by long chain fatty acid. Foam can be generated by bubbling air through a fritted glass column containing glycerol, water, lipase and fatty acid (figure 1.6). This results in the

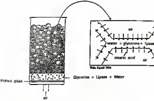


Figure 1 6 Schematic representation of a foam column and a thin liquid film around a bubble formation of an air/water interface stabilized by the fatty acid molecules. The fatty acid forms a complex with the lipase and this complex then reacts with glycerol molecules to form an ester bond giving diglyceride and triglyceride

CHAPTER 2
EXPERIMENTAL

2.1 Introduction

This chapter contains detailed information about the materials and experimental procedures used in this dissertation. The following chapter will mention specific experimental protocols employed and will refer to this chapter for details of the experiment.

2.2 Materials

The surfactants used in this document were Diethylhexyl sodium sulfosuccinate (Aerosol-OT or AOT) and Na-oleate. The AOT was purchased from Sigma Chemical Inc (St. Louis, MO) and Na-oleate from Fluka (Germany). Both surfactants were 99% pure and were stored in a desiccator. Most chemicals used, including organic solvents, alcohol and fatty acid substrates, and buffer salts, were of high purity (>99%) and were stored as stated in the instructions and employed without any further purification. Standards (monoglycerides, diglycerides, triglycerides and fatty acids) for High Performance Liquid Chromatography were purchased from Sigma Chemical Inc (St. Louis, MO). Double distilled deionized water was used throughout.

The enzyme lipase, called Lipozyme 10 000L (lot # 705, batch LMN 0006), a product of Novo Laboratory Inc., was made available by Kraft Research Inc. (Glenview, IL) Lipozyme is a fungal lipase produced by submerged fermentation of a selected strain of *Mucor miehei*. It is a brown liquid having a density of 1.15g/ml. It was used without further purification so that the microemulsion formulation developed could be used directly for scale-up process It has molecular weight of 33,000, with isoelectric pH of 7.6 and has 1,3 positional specificity having optimum activity for lauric acid, its activity decreases with increase in chain length of fatty acid. Other properties of this enzyme are contained in detail in other sources [150, 151]

2.3 Methods

2.3.1 Procedure for Enzymatic Reactions in Microemulsions

Lipase was incorporated into reverse micellar media by the method of injection. First, a reverse micellar media composed of either AOT or Na-oleate and t-butanol, plus fatty acid, glycerol, and a bulk hydrocarbon, usually isooctane-(2,2,4-trimethylpentane), was formed in a 50 ml conical flask (Figure 2.1) It was stirred using a magnetic stirrer to make the media clear, i.e. to achieve solubilization of glycerol in a water-in-oil microemulsion. The concentrations of surfactant and substrate were chosen so that the system remains in the w/o microemulsion region even after the addition of lipozyme solution. Lipozyme solution contained phosphate buffer at a pH of 7 and the unpurified lipase. When the effect of pH was studied, where buffer solutions of various pH were added All the reactions were conducted at room temperature (23 ± 2° C), unless otherwise stated Microemulsions were

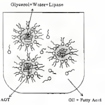


Figure 2.1 Schematic representation of w/o AOT microemulsion with glycerol, water and lipase in aqueous core and fatty acid in oil phase

maintained at different temperatures, to study the effect of temperature by immersing the conical flask in a large beaker containing water and placing this beaker on a hot plate. The water temperature was monitored using a thermometer and a magnetic bar was used for stirring. Samples from the microemulsion were taken after regular time intervals for kinetic analysis. Reaction kinetics were monitored by the titration of fatty acid using 0.02 M NaOH in ethanol, thin layer chromatography, and HPLC.

2.3.2 Thin Layer Chromatography

Thin Layer Chromatography (TLC) plates were run twice in diethyl ether up to 2 cm, and then in a hexane/diethyl ether/acetic acid mixture (70/30/1 v/v/v). Then diluted sulfuric acid (10% by volume) was spread on the plate and the plate was heated in an oven at 400° C for visualization of spots. In some cases, spots were revealed in an iodine vapor chamber.

2.3.3 High Performance Liquid Chromatography

Qualitative and quantitative analysis of products of enzymatic reactions were done using High Performance Liquid Chromatography (HPLC) (Figure 2.2). The system is fully computerized using a 640 kB Epson Equity I+ running DoubleDos and Spectra Physics software LNET2 and SPMENU. Once programmed, this system can analyze over 80 samples without operator assistance. The system can inject a sample taken from the reaction media, identify the components in the sample, determine the concentration of each component (integrate the signal), store all

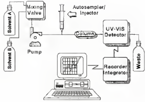


Figure 2.2 Schematic representation of HPLC instrumentation and set-up

information (including the raw data), generate a file for a spreadsheet, and print a final report. Although the system has several components, the principles of operation are relatively simple. To achieve separation, HPLC uses the relative affinity of a compound between a polar and a nonpolar phase. A nonpolar solvent is pumped through a separation column containing silica. The retention time in the column depends on the compound's affinities for the two phases. Consequently, depending on their polarity and functionality, two or more compounds in the same sample separate as they pass through the column. This type of chromatography is called "normal-phase" chromatography.

After separation, the compounds in the eluent flow through a UV/VIS absorbance detector (Spectra Physics Model SP8450) which generates an electrical signal proportional to the light absorbed by the liquid passing through the detector. Peaks in plots of this signal correspond to compounds eluting from the column. The retention time for various compounds will be different if column selection, eluent selection, and flow rate are chosen carefully.

The other components of the HPLC system are the pump, autosampler, and integrator. The pump (Spectra Physics Model SP8800) maintains the flow of carrier solvent through the system at a steady rate. The pump can also mix up to three miscible solvents in any ratio.

The autosampler (Spectra Physics Model SP8880) contains four trays for sample vials mounted on a turntable. Each tray holds twenty vials and the turntable contains an additional priority-vial position. The autosampler moves each vial to the sampling position and, when the system is ready, injects a predetermined volume of the contents into the solvent stream while signalling the other instruments to begin analysis.

The integrator (Spectra Physics Model SP4290) plots the raw signal from the detector (optical absorbance of the solvent stream) and determines the presence of peaks. The integrator calculates the area of peaks and determines if they correspond to programmed compounds. If a peak is identified as a programmed compound, previously entered calibration data are used to determine the amount of the compound detected. A good description of analysis of glycerides by normal phase HPLC is given elsewhere [152].

2.3.4 Quasi-Elastic Light Scattering

Quasi-elastic light scattering (QELS) was used to monitor the variation of hydrodynamic radius of reverse micelles during reaction (Figure 2.3). The optical source for the light scattering apparatus was a Spectra Physics argon ion laser model 2000 operating at 514.5 nm. Whenever a micelle entered the path of light, it caused the light to scatter in all directions. The scattered light was collected at an angle of 90 degree using a laser light scattering goniometer (Brookhaven Instruments model 200SM) then was focused on a photomultiplier cathode (Brookhaven Instruments EMI-9865). The time dependent correlation function of the scattered intensity was recorded by using a fast clipped real time correlator (Brookhaven Instruments model BI 2030) with 64 channels and a time resolution of 100 ns. The normalized

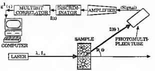


Figure 2.3 Schematic representation of quasi-elastic light scattering instrument.

correlation function of the scattered electric field $g^{(1)}(t)$ was analyzed by using the cumulant method [153,154] in which the logarithm of the normalized $g^{(1)}(t)$ was fitted to a polynomial equation using a nonlinear least-squares fitting routine. The fitting equation is:

$$\ln[(c_1 b)^{1/2} g^{(1)}(t)] = \ln(c_1 b)^{1/2} - \Gamma t + \mu_2(t^2/2) \qquad [2.1]$$

where c_1 and b are the baseline and the instrument constant. The first cumulant of line width distribution, Γ, is Dq^2, where D is the time averaged diffusion coefficient and q is magnitude of scattering vector: $q = (4\pi n/\lambda_o) \sin(\theta/2)$, where n is the refractive index of continuous medium, λ_o is the wavelength of light in vacuum, and θ is the scattering angle. The second cumulant, μ_2, provides the polydispersity of the line width distribution. The polydispersity, Q is defined as $Q = \mu_2/\Gamma^2 = (D^2_a - D^2)/d^2$ The hydrodynamic radius was calculated from the well known Stokes-Einstein equation, $R_H = kT/(6\pi\eta D)$, where k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the continuous medium. This equation assumes spherical shapes for suspended particulates which undergo Brownian motion The diffusion coefficient D is at infinite dilution.

2.3.5 Self-diffusion Measurements

Self-diffusion coefficients were obtained using the ^{1}H-NMR Fourier transform pulsed-gradient spin-echo (FT-PGSE) technique with a standard JEOL FX-100 NMR spectrometer operating at a proton frequency of 99.6 MHz and a temperature of 25 ± 0.5°C. The PGSE measurements, taken by varying the duration of the gradient pulse (1 Gauss cm^{-1}) at a constant measuring time of 140 ms were performed as follows First, the self-diffusion coefficient of D_2O was measured in isooctane. Various microemulsion samples were made using D_2O instead of water. The self diffusion coefficient of D_2O in microemulsions was measured as described by Stejskal and Tanner [155]

Intermolecular contributions to spin relaxation processes originate in part from the modulation of dipole-dipole interactions through translational diffusion. They are significant for protons in solution and electron-nuclear dipole relaxation in paramagnetic systems. In the PGSE technique, the basic magnetic field is essentially homogeneous throughout the experiment. The effective dispersion of refocussing of the spins occurs in two identical field gradient pulses The diffusional attenuation of the echo amplitude is given by Stejskal and Tanner [155]

$$A/A_o = \exp[-\tau^2 G^2 D\, \delta^2 (\Delta - \delta/3)]$$

where A/A_o is the ratio of echo amplitudes in the presence and absence of gradient, τ is the gyromagnetic ratio, G is the magnitude of gradient, δ is the duration of each gradient pulse, and Δ is the time between the two gradient pulses (corresponding to the diffusion time) The self-diffusion coefficient of water in microemulsion (D_w) and the self-diffusion coefficient of water in isooctane (D^o_w) gives the idea of the structure of the water in the microemulsion When the self diffusion coefficient of water in the microemulsion (D_w) is one hundredths of the self-diffusion coefficient of water in isooctane (D^o_w), then microemulsion is water in-oil type When the-self-diffusion coefficient of water in the microemulsion (D_w) is one tenth of the-self diffusion coefficient of water in isooctane (D°_{w}), then microemulsion is bicontinuous in nature [155].

CHAPTER 3
ESTERIFICATION IN AOT MICROEMULSIONS

3.1 Introduction

A microemulsion may be defined as a thermodynamically stable isotropic dispersion of two immiscible liquids consisting of microdomains of one or both liquids, stabilized by an interfacial film of surface active molecules [65,156]. Microstructural studies of microemulsions have been given considerable attention because of their interesting physicochemical properties and their potential for various applications of commercial importance [21,157-160]. Much of this effort has been directed toward elucidating the type of fluid microstructures present in microemulsions and the changes which take place as the relative amounts of oil and water vary, particularly for microemulsions that progressively invert from oil-continuous to water-continuous type. The structure of microemulsions has been investigated by a variety of techniques including electrical conductivity, NMR spectroscopy, self diffusion measurements, quasi-elastic light scattering, small angle neutron and x-ray scattering, and optical spectroscopy [161-170]. A microemulsion containing a relatively low fraction of oil confined within small isolated droplets and dispersed in water, is known as an oil-in water (o/w) microemulsion, while the reverse type (small amounts of water dispersed in large amounts of oil) is a water-in-oil (w/o) microemulsion. If one continuously increases the water to oil ratio in a w/o microemulsion, phase inversion ultimately may occur. During such an inversion, an intermediate, transparent, isotropic, bicontinuous structure may form consisting of both oil- and water continuous domains, separated by interfacial surfactant layers. On the other hand, in certain systems a sharp transition from well-defined w/o to well-defined o/w structure (without the intermediate formation of a bicontinuous phase) is believed to occur.

The development of molecular enzymology has come about mainly through studies of free enzymes. Experiments can be designed to elucidate the structure of the catalytic site and the physicochemical conditions for its optimal activity. The use of low water content microemulsions as a medium for enzymic reactions has been reported by many researchers [124,171-176]. The majority of work reported in the literature is primarily concerned with the usage of the enzyme lipase. It has been demonstrated that the lipase retains a high intrinsic activity in these highly ordered non-polar solutions (i.e. water-in-oil microemulsions). Immobilized enzymes can also be used in a microemulsion medium without too much impairment of the reaction rate. The anionic double-tailed surfactant AOT (sodium di-[2-ethylhexyl] sulfosuccinate), is most frequently used to form microemulsions. Unlike most surfactants, AOT does not require additional amphiphiles as cosurfactants for the formation of reverse micelles because its wedge-shaped molecular geometry. To date esterification reactions in microemulsions reported in the literature have been based on the expensive and pure lipase from *R. delemer* and *C. cylindracea* [124,177,178]. We have designed an experiment using a commercially used lipase called Lipozyme

(10,000 LU/g) We have investigated all possible physicochemical conditions such as oil chain length of continuous phase, glycerol/water ratio, chain length of fatty acid, oleic acid/glycerol ratio, water/enzyme ratio and unsaturation of fatty acid which can influence the synthesis reaction.

3.2 Experimental Procedure

Lipozyme, a fungal lipase produced by Novo Nordisk, Denmark was made available by Kraft Research Inc (Glenview, IL) Hexane, iso-octane, iso-propanol (all HPLC grade) and buffer solutions were bought from Fisher Scientific Company Fatty Acids (C_8 -C_{20}, 99% pure), Lipids (99% pure) and AOT (99% pure) were purchased from Sigma Chemical Company. All the chemicals were used as supplied Deionized double distilled water was used in all the chemical procedures All experiments were repeated three times and results were found to lie within a range of +/- 2%.

3.2.1 Reactions in Microemulsions

Microemulsions were prepared by mixing the alkanes, surfactant, glycerol, lipozyme, and water in the required proportions, under constant stirring, yielding a transparent microemulsion Subsequently, the desired amount of a fatty acid was added to the microemulsion. The amounts of individual components of the microemulsion are mentioned individually in results and discussion section. The addition of fatty acids to the microemulsion initiates the esterification reaction. The extent of reaction was monitored by NaOH titration in nonaqueous medium using phenolphthalein as an indicator. The quantitative analysis of products was carried out using normal phase HPLC and TLC.

The synthesis reaction taking place in the microemulsion is:

$$\text{Glycerol} + \text{Fatty Acid} \quad \overset{\text{Lipase}}{=\!=\!=\!=\!=\!=\!=\!=\!=>} \quad \begin{bmatrix} \text{Monoglyceride} \\ \text{Diglyceride} \\ \text{Triglyceride} \end{bmatrix} \quad + \text{ Water}$$

3.2.2 Monitoring the Reaction Using HPLC and TLC

Normal phase HPLC with a UV absorbance detector having a cut-off wavelength of 213 nm and 0.05 absorbance units full-scale (AUFS) was used to separate monoglyceride, diglyceride, triglyceride, and fatty acid in order to monitor the reaction. The silica column (10 mm x 2.4 mm) and the mixture of iso-octane and iso-propanol (94/6, v/v) as a mobile phase were used at room temperature. The flow rate of mobile phase was kept at 1 ml/min [179]. The integrator ploted the raw signal from the detector and determined the presence of peaks, retention times, and quantities of various species. Standard samples of triolein, diolein, monoolein, and oleic acid were dissolved separately in the eluent (0.001gm/ml) then filtered through a 0.2 μm filter. The autosampler was used to inject 20 μl of sample each time. First, residence times of triolein, oleic acid, diolein, and monoolein were individually identified. Then, a mixture of the above lipids and oleic acid were run three times to check the reproducibility of the technique. The sample from the microemulsion containing AOT was diluted ten times with the eluent before injecting into the column. Due to the adsorption of AOT in the column, slight shifts in the residence times of the various species were observed. The standard and the sample from the microemulsion were injected several times to confirm reproducibility of residence times and areas under the peaks. The external standard method was used on the integrator to find the actual weight percent of products in the sample from the microemulsion. With external standards each peak of interest in the analysis sample was compared with the same peak in the calibration sample. Because the amount of each component in the calibration sample was known, a ratio between that component and the analysis sample could be calculated, giving the amount of the component in the analysis sample.

TLC plates were run twice in diethyl ether up to 2 cm, and then in a hexane/diethyl ether/acetic acid mixture (70/30/1, v/v/v).

3.3 Results and discussion

3.3.1 Solubilization of Glycerol/Water in AOT and Hexane

Mixtures of glycerol and water in various proportions were titrated separately in 5 ml of 0.25 M solutions of AOT in hexane to study the effect of glycerol/water ratio on the solubilization capacity of these microemulsion systems. The maximum solubilization was taken as the point where the onset of turbidity took place. The solubilization capacities per ml of the microemulsion for various mole fractions of glycerol in water are plotted in Figure 3-1. The result shows a maximum at a mole

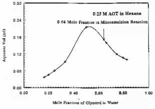


Figure 3-1 Effect of mole fraction of glycerol to water on their solubilization in the AOT + hexane solution.

fraction of glycerol to water 0.5 (Figure 3.1). Before this maximum, glycerol molecules get solubilized near the hydrocarbon chain of AOT by pushing the water molecules inside the aqueous core, and at this maximum, the hydrocarbon chain of AOT became fully saturated with glycerol molecules. Upon further increase of the mole fraction, glycerol molecules began solubilizing in the aqueous core, causing a decrease in the ionization of AOT molecules and hence, a decrease in the total solubility. This in turn decreases the repulsive forces between the ionic groups causing a decrease in the solubilization of the aqueous phase. However the synthesis reaction was found to be optimum at 0.64 mole fraction of glycerol because more free water shifts the equilibrium towards hydrolysis and less free water results in inactivation of the lipase.

3.3.2 Effect of Oil Chain Length on Reaction Kinetics

In this experiment, various alkanes (C_6 - C_{16}) were taken as the oil phase for making the microemulsions and percent conversion of oleic acid was monitored by NaOH titration. The AOT concentration was 0.53 M in the oil. The oil chain length had an interesting effect on the reaction. The conversion increases with chain length (from hexane to hexadecane) after one hour but for equilibrium conversion the reverse was true, as shown by data after 9 hours of reaction (Figure 3.2). This is because there are two rate controlling steps in this reaction system. First there is the inter-droplet interaction, which was maximum for the hexadecane microemulsion system [170]. Second there is the diffusion of products (e. g. monoglyceride,

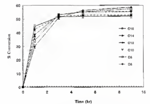


Figure 3.2 Effect of oil chain length of continuous phase on % conversion of oleic acid.

triglyceride) from the interface to the bulk oil phase, which was maximum in the hexane system. Chao and Shah reported that in the oil/water/surfactant system, surfactant partitioning in the oil phase decreases as the oil chain length increases [219] From the consideration of geometric packing of surfactants at the interface, longer chain length of oil has more difficulty in penetrating into the surfactant layer [170] The magnitude of attraction between droplets increases with increase in the difference between the compositions of interface and the continuous phase The greater difficulty in penetrating the interface by larger oil molecules would increase this difference, thus increasing the magnitude of interaction. The larger the interaction, the greater is the likelihood of droplets containing enzyme to collide with other droplets containing glycerol or enzyme This collision caused exchange of glycerol between enzyme containing and empty droplets In this system only 8% of the microemulsion dropletes contained enzyme molecules. When a droplet containing glycerol and enzyme comes in contact with oleic acid, then, reaction takes place So the distribution process of glycerol into an enzyme containing droplet plays an important role in the beginning which causes a difference of 15 percent conversion from hexane to hexadecane system in the first hour (Figure 3 2) As the reaction is continued for three hours, diffusion of products from the interface to the bulk plays a vital role in controlling the reaction. Hence, the equilibrium conversion of oleic acid into products in hexane (low viscosity) was 58%, and in hexadecane (high viscosity) the equilibrium conversion was 50% after 10 hours of reaction (Figure 3 2)

3.3.3 Effect of Fatty Acid Chain Length on the Reaction Kinetics

For this experiment, we used microemulsions with 0.53 M AOT in hexane as the oil phase. The glycerol/fatty acid ratio was kept constant at 10:1 in order to dissolve the long chain fatty acids in hexane. Table 3.1 showed the absolute amounts of the various fatty acid added in 7 ml of AOT microemulsion solutions. The results shown in Figure 3.3 demonstrate that the conversion of fatty acids into glycerides increased with increased fatty acid chain length. Maximum conversion was obtained using by arachidic (C_{20}) acid. Due to its longer hydrocarbon chain, the product of C_{20} acid was more nonpolar than the products of other fatty acids. The product partitioned preferentially into the oil phase after it formed allowing more C_{20} acid to come to the interface and react at the same site. Here the dominant factor controlling the reaction was the partitioning of products into oil phase which was maximum for C_{20} acid and minimum for C_{10} acid.

Table 3.1 The absolute amounts of various fatty acid added in 7 ml of AOT microemulsion to maintain glycerol/fatty acid ratio of 10:1.

Fatty Acid	Amount of fatty acid in 7 ml of microemulsion
C_{10} Acid	0.1347 gm
C_{14} Acid	0.1783 gm
C_{16} Acid	0.2002 gm
C_{18} Acid	0.2222 gm
C_{20} Acid	0.2440 gm

3.3.4 Effect of Oleic Acid/Glycerol Ratio on the Reaction Kinetics

In this experiment, we used microemulsions with 0.53 M AOT in hexane as the oil phase, while oleic acid/glycerol ratio was varied. Glycerol was kept constant

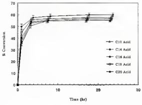


Figure 3.3 Effect of fatty acid chain length on its % conversion with continuous phase as hexane.

and amounts of oleic acid was increased This showed a maximum equilibrium percent conversion at 1:1 molar ratio of oleic acid to glycerol (Figure 3 4) We found that for molar ratios greater than 1, an increase in oleic acid at the interface had a significant effect in decreasing the diameter of the microemulsion droplets which ultimately decreased the conversion by exposing part of the enzyme into the oil phase As the reaction proceeded, the volume of aqueous phase decreased because the molar volume of glycerol is 62 46 ml/mole whereas the molar volume of water is 18 ml/mole If one mole of glycerol is converted into monoolein, then one mole of water is formed. So, net loss of aqueous volume is 44 46 ml (62.46 - 1 x 18) Similarly, if diolein is formed, then net loss of aqueous volume is 26.46 ml (62.46 - 2 x 18) If triolein is formed, then net loss of aqueous volume is 8.46 ml (62.46 - 3 x 18). In any case there is loss of aqueous volume Other possible reason may be that the enzyme-catalyzed reaction is diminished by the excess of substrate This well known phenomenon is called substrate inhibition.

3.3.5 Effect of Subsequent Addition of Glycerol or Water after Equilibrium is Reached

Brozozowski [180] showed that the active site of the enzyme is close to the surface but not completely exposed to the solvent It is buried under the head of a long loop (lid) folded onto the triad and stabilized by extensive hydrophobic and electrostatic interactions Thus, an enzyme could be working in two stages. In one stage the lid is removed or displaced, possibly through interfacial activation, so the

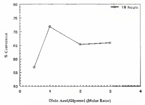


Figure 3.4 Effect of oleic acid/glycerol ratio on the equilibrium % conversion after 18 hours (Glycerol concentration was kept constant in all experiments shown here)

ester bond can be subsequently hydrolyzed or synthesized. In other stage, the lid also serves as a device to inhibit the proteolytic activity of the triad, thereby protecting the enzyme itself. Due to the consumption of glycerol, the size of the droplet decreases as synthesis proceeds. At a certain point, the droplet size becomes too small to accommodate the enzyme and this may cause the lid to close. The addition of extra glycerol can reactivate the enzyme since it may now move out of the oil phase and into the interface and the lid may reopen. Figure 3.5 shows the results of an increase in percent conversion of oleic acid from 50 to 58% after the addition of glycerol. The glycerol to oleic acid ratio after addition of glycerol was 2.1:1. The increase in conversion was not due to an increase in glycerol (substrate) concentration because the calculated ratio glycerol to oleic acid ratio after equilibrium (before the addition of glycerol) was 1.7:1 and Figure 3.4 indicates that this ratio of glycerol to oleic acid was sufficient for synthesis reaction. The addition of water enlarges the droplets. As the droplets enlarge, thus reactivating the enzyme. However, addition of water decreases the percent conversion (Figure 3.5) as the enzyme starts to catalyze the hydrolysis of the mono- and diglycerides formed during the synthesis reaction. This shows that the enzyme is indeed active even after equilibrium has been achieved.

3.3.6 Effect of Water/Enzyme Ratio on Equilibrium Conversion

The enzyme was freeze-dried to remove all associated water. The Lipozyme as delivered from the supplier contains 30% water by weight. After all possible

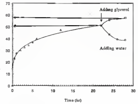


Figure 3.5 Effect of addition of glycerol or water on % conversion after first equilibrium.

water was removed increasing amounts of water were added to it to study the effect of water content on equilibrium conversion. Here amount of enzyme was kept constant. A maximum in the equilibrium conversion was found at a water content near 30% (Figure 3.6). This 30% water is bound to enzyme molecules and AOT molecules in the microemulsion. A further increase in water induced accumulation of free water inside the aqueous core, free water that caused hydrolysis of ester bonds. The equilibrium percent conversion decreased at higher concentrations of water in microemulsions reaching zero conversion at 1.10 (w/w) with respect to the enzyme

Let us consider the law of mass action of the following reaction

$$\text{Glycerol} + \text{Fatty Acid} \rightleftharpoons \text{Monoglyceride} + H_2O$$

$$K_c = [\text{Monoglyceride}][H_2O]/[\text{Glycerol}][\text{Fatty Acid}]$$

Here [ ] represents concentrations of reactants or products. The value of equilibrium constant (K_c) of above reaction is fixed for a given temperature, pressure and enzyme concentration. If concentration of water is increased when reaction is at equilibrium, the reaction will proceed in the reverse direction in order to maintain K_c. This will cause a decrease in concentration of monoglyceride and an increase in the concentration of glycerol and fatty acid. Similar will be the case for diglyceride or triglyceride. Therefore, due to high water content, the conversion was zero at 1.10 of water/enzyme ratio (Figure 3.6)

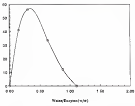


Figure 3.6 Effect of water/lipozyme ratio on the % conversion of oleic acid in microemulsions mediated reactions.

3.3.7 Effect of Unsaturation of Fatty Acid on the Reaction Kinetics

The structure of the fatty acid had a profound effect on the percent conversion of fatty acid to mono- and diglycerides in microemulsions (Figure 3.7). A maximum conversion of 84% was found in the case of linoleic acid, 80% for linolenic acid, 71% for oleic acid, and 33% for stearic acid. This is believed to be due to the fluidity of interface caused by unsaturation in the fatty acid. An increase in fluidity caused an increase in the rate of the reaction. Also, as the unsaturation in fatty acid increased, its solubility in hexane also increased. Because stearic acid is slightly soluble in acetone, oleic acid is more soluble in acetone as compared to stearic acid whereas linoleic acid and linolenic acid are highly soluble in acetone as indicated in the CRC handbook of Chemistry and Physics. This indicates that unsaturation in C_{18} fatty acid increases its solubility in nonpolar medium. This increase in solubility from stearic acid to linoleic acid gave the products at the interface a driving force to move them quickly into the oil phase, resulting in an increase in percent conversion. The very small difference in the percent conversion for linoleic acid and linolenic acid was within experimental error. It seemd that an increase in unsaturation from two to three double bonds had the least effect on the difference in their percent conversion. Although unsaturation was higher for linolenic acid as compared to linoleic acid, the differnce in the partitioning of products from these two fatty acids is very low.

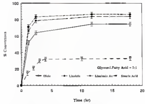


Figure 3.7 Effect of unsaturation of fatty acid on its % conversion.

3.3.8 HPLC Analysis of Products from w/o Microemulsion

Samples from microemulsion systems were mixed with equal volumes of ethanol for HPLC analysis using the external standard method. These studies showed that monoolein (42% by weight) and diolein (21% by weight) are the only products formed during the enzymic reaction of oleic acid and glycerol in microemulsions (Table 3.1). Table 3.1 also shows equivalent of oleic acid present in the samples from AOT microemulsion in monoolein, diolein, and unreacted oleic acid. The total amount of oleic acid taken was 0.004761 moles. The total equivalent of moles of oleic acid from all the products after reaction is 0.004751 which is in excellent agreement with the initial amount of oleic acid taken (0.004761 moles). The conversion of oleic acid is 63% after the reaction reached equilibrium in 18 hours. This was a much higher conversion than that reported by Ergan et al. [181] who found 22% diolein, 11% monoolein, and 5% triolein after 60 hours reaction in bulk aqueous media. We believe that diolein diffused from the interface to the oil phase due to its high partitioning in the oil phase. Hence no triolein formation was observed. Similar esterification reaction was carried out in bulk phase (no alkane) by Schuch et al. [182]. They observed the formation of triolein. This means that the microemulsion system allows diolein to diffuse to the oil phase. Therefore, one can use microemulsions for selective synthesis of monoolein and diolein.

Table 3.1 HPLC analysis of product from esterification of glycerol and oleic acid in AOT microemulsions

	HPLC	Equiv. moles of oleic acid	NaOH titration
Monoolein	42 % (by wt.)	0.001930	
Diolein	21 % (by wt.)	0.001060	-
Triolein	0	0.000000	-
Oleic Acid	37 % (by wt.)	0.001761	43 % (by wt.)

Total equivalent moles of oleic acid 0.004751

3.3.9 Effect of Esterification on the Average Size of Droplets

The average size of reacting microemulsion droplets was measured using QELS at various time intervals (Figure 3.8). As we saw in section 3.3.4, there was a net decrease in the aqueous volume due to esterification. It clearly shows a decrease in the diameter from 95 Å to 58 Å after four hours when reaction reaches to equilibrium. The unit cell of *M. miehei* triacylglycerol lipase has been mapped and has dimensions of a=71.6 Å, b=75.0 Å and c= 55.0 Å [180]. It is evident that the enzyme must have been partly outside the aqueous cores. The reaction reached equilibrium due to the increase in the water content in aqueous core and squeezing out of the enzyme from droplets. This measurement showed that QELS can be used to monitor reaction in order to maintain optimum size for optimum reaction in microemulsions.

In summary, the synthesis reaction was found to be optimum at 0.64 mole fraction of glycerol to water because additional water shifts the equilibrium towards hydrolysis. The conversion increases with chain length (from hexane to hexadecane) after one hour but for equilibrium conversion after 10 hours the reverse is true. The conversion of fatty acids into glycerides increased with increased fatty acid chain length A maximum equilibrium percent conversion at 1:1 molar ratio of oleic acid to glycerol was observed A maximum conversion of 84% was found in the case of linoleic acid, 80% for linolenic acid, 71% for oleic acid, and 33% for stearic acid The HPLC analysis showed the presence of monoglycerides and diglycerides but no triglyceride in the products from AOT stabilized water-in-oil microemulsions.

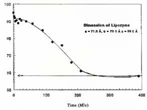


Figure 3.8 Change in the average size of microemulsions during reactions.

CHAPTER 4
ESTERIFICATION IN MONOLAYERS AND AOT MICROEMULSIONS

4.1 Introduction

Lipases are enzymes that catalyze the hydrolysis or synthesis of ester bonds. Optimal activity of lipase is observed in the presence of an interface. The best studied lipases are those that are water soluble using substrates that are water insoluble. Thus, monolayers and microemulsions are deemed suitable media to study the effect of curvature of the interface, fluidity of the interface, pH of the aqueous phase, and the residence time of reactants and products at the interface on enzymatic reactions.

The hydrolysis of lipid monolayers has been studied extensively for various substrates using different enzymes [145,146-148,183-185]. However, very little work has been done on the enzymatic synthesis in monolayers. Synthesis in monolayers is of great interest because of specific molecular orientation of polar and nonpolar groups as well as high surface concentration of substrate molecules. In addition, factors such as molecular area, surface charge density, and surface fluidity can be varied at will in monolayers.

The hydrolysis of lecithin monolayers by phospholipase A has been studied by measuring the decrease in surface potential [146]. Several types of phospholipases that catalyze the hydrolysis of acyl-ester bonds in monolayers of glycerophosphatidyles have also been reported [148,183]. It was observed that the charge on the substrate surface is important to the initiation of the enzymatic reaction. Shah and Schulman [145] measured the action of snake venom phospholipase A on lecithin monolayers. They investigated the influence of divalent cations, various buffers, and the presence of other lipids on the hydrolysis of dipalmitoyl, egg, soybean, and dioleoyl lecithin monolayers. It was shown that a lecithin monolayer does not hydrolyze when it was compressed above a critical surface pressure. The critical surface pressure required to block the penetration of the enzyme into the monolayer increased with molecular area (i.e. dioleoyl lecithin > soybean lecithin > egg lecithin > dipalmitoyl lecithin). Colacicco [184] has reported measurement of surface potential was important to monitor enzymatic reactions of phospholipid monolayers [184]. Thus, monolayers represent a promising system to study the synthesis reaction of stearic acid (as monolayer) and glycerol (in subphase) using Lipozyme (10,000 L Novo Enzyme).

Enzymatic reactions in microemulsions with low water content has been the topic of interest for several groups [122,124,171-174]. The anionic double-tailed surfactant AOT (sodium di-[2- ethylhexyl] sulfosuccinate) is most frequently used to form microemulsions. Unlike most surfactants, AOT does not require additional amphiphiles as cosurfactants for the formation of reverse micelles because of its wedge-shaped molecular geometry. The rate of enzymatic reaction in reverse micelles depends on surfactant concentration, water to surfactant ratio, temperature, concentration of buffers, and pH. The enzymatic synthesis of mono-, di-, and triglycerides has been studied to understand the mechanism of reaction between oil-soluble fatty acid and water soluble glycerol by the enzyme (lipase) in reverse micellar solutions [144,186].

4.2 Experimental Procedure

4.2.1 Monolayer Formation

Stearic acid dissolved in a solvent (hexane:methanol:chloroform at 3:1:1, v/v/v) was spread on a subsolution of water and glycerol using an Agla micrometer syringe. After the evaporation of the solvent, the monolayer was compressed with a waxed glass bar up to the desired initial surface pressure. When the surface pressure reached a steady value, lipozyme was injected under the monolayer and stirred gently by a rolling magnet. This assured the uniform distribution of the enzyme in the subsolution. The surface pressure and surface potential were measured with time. After three hours, the monolayer was removed by suction and the product was dissolved in a solvent of methanol:chloroform (2:1, v/v). The sample for HPLC was taken from the methanol - chloroform solvent phase.

4.2.2 Microemulsion Formulation

A typical microemulsion consisted of 14 ml of alkane and 0.004761 moles of fatty acid in the oil phase with 3.3 g of AOT as the surfactant, and 0.010043 moles of glycerol, 0.1 ml of lipozyme, and water in the aqueous phase. First glycerol, lipozyme and water were solubilized in 14 ml of alkane containing 3.3 g of AOT, then fatty acid was added to start the reaction. The microemulsion was prepared by mixing the above contents using constant stirring. The reaction was monitored with NaOH titration using phenolphthalein as the indicator for the extent of reaction and by HPLC for quantitative analysis of products formed.

4.2.3 Reaction to be Investigated

The synthesis reaction taking place in the microemulsion and monolayer is:

$$Glycerol + Fatty\,Acid \xrightarrow{Lipase} \begin{bmatrix} Monoglyceride \\ Diglyceride \\ Triglyceride \end{bmatrix} + Water$$

4.2.4 Monitoring the Reaction by HPLC and TLC

Normal phase HPLC on a silica column (10 mm x 2.4 mm) with a UV absorbance detector of 213 nm cut-off wavelength and 0.05 absorbance units full-scale (aufs) was used to separate monoglyceride, diglyceride, triglyceride, and fatty acid to monitor the reaction. A mixture of iso-octane and iso-propanol (94/6, v/v) was used as the mobile phase. The flow rate of the mobile phase was kept at 1 ml/min. All analyses were done at room temperature. The integrator plotted raw signals from the detector and determined the presence of peaks.

TLC plates were run twice in diethyl ether up to 2 cm, and then in hexane/diethyl ether/acetic acid mixture (70/30/1, v/v/v)

4.3 Results

4.3.1 Reactions in Monolayers

A monolayer of stearic acid was spread on a subsolution of glycerol and water (1:1, v/v; total volume = 85 ml at pH 6.8). The monolayer was compressed to the desired surface pressure (22 dynes/cm) and lipozyme solution was injected under the monolayer. Lipozyme was gently mixed under the monolayer by rolling the magnetic bar. The surface pressure and surface potential were measured with time as described earlier [188]. It was evident that surface pressure remained constant (Figure 4.1) whereas surface potential increased by about 90 mV (Figure 4.2). The synthesis reaction was carried out at three concentrations of lipozyme (144 LU, 216 LU, & 288 LU per ml of subsolution). No change in the surface potential of stearic acid monolayer was observed without lipozyme. In order to establish that the change in the surface potential was indeed due to the formation of ester bonds, we carried out additional experiments using a monolayer of stearyl alcohol instead of stearic acid but under the same conditions as before. There was no change in the surface potential after injecting the lipozyme under the stearyl alcohol monolayer. On the other hand, because of cleavage of the ester bond in lecithin monolayers, there was a decrease in the surface potential [145,184]. The change in surface potential of the monolayer was measured at various initial surface pressures to determine the affect of surface pressure on the synthesis reaction (Figure 4.3). It is evident from the results shown in Figure 4.3 that an initial surface pressure of 20 dynes/cm was

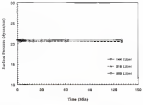


Figure 4.1 Surface pressure - time curves for enzymatic reactions in stearic acid monolayers at various lipozyme solution.

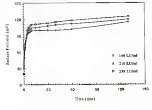


Figure 4.2 Surface potential - time curves for enzymatic reactions in stearic acid monolayers at various lipozyme concentrations. The initial surface pressure was 22 dynes/cm for each monolayer

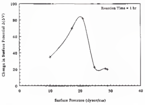


Figure 4.3 Effect of initial surface pressure on the change in surface potential of the stearic acid monolayers in 1 hour

optimal for maximum change in the surface potential (i.e., maximum conversion). Initially the change in surface potential increased with the increase in the surface pressure. It was mainly due to the increase in the two dimensional concentration of reactant at the air/water interface. After reaching the optimum surface pressure of 20 dynes/cm, the change in the surface potential decreased with an increase in initial surface pressure, because stearic acid molecules were so tightly packed that it reduced the penetration of the active site of the enzyme in the monolayer and hence the rate of reaction.

The product from the monolayer was analyzed to confirm the synthesis reaction. After the completion of the reaction, the monolayer was removed and mixed with chloroform and methanol (1:2 v/v) solvent to dissolve possible mono-, di- and triglycerides. Performing HPLC on this sample indicated the presence of tristearin (32.6 %), monostearin (55.7 %), and stearic acid (11.7 %) in the monolayer. Performing TLC on the extracted monolayer also confirmed the presence of these products. It should be noted that both TLC and HPLC showed the absence of distearin in the product from monolayer. In contrast, the product from microemulsion had monostearin and distearin.

4.3.2 Reactions in Microemulsions

The structure of the fatty acid had a profound effect on the percent conversion of fatty acid in microemulsions (Figure 3.6). A maximum conversion of 84% was found in the case of linoleic acid, 80% for linolenic acid, 71% for oleic acid, and 33.4% for stearic acid. This occurs due to the change in partitioning of a fatty acid between the interface and the oil, caused by unsaturation of the fatty acid. As unsaturation of a fatty acid increases, its solubility in hexane increases. This increase in solubility from stearic acid to linoleic acid gives a driving force to the products at the interface to move quickly into the oil phase. This results in an increase in percent conversion. There was a very small difference between linoleic acid and linolenic acid because both of these fatty acids are unsaturated, and although unsaturation is more for linolenic acid, the difference in solubility of the products in hexane is presumably very low. Analysis by HPLC of a sample from microemulsion containing stearic acid indicated the presence of monostearin (12.4%), distearin (21.0%) and stearic acid (66.6%). In contrast to stearic acid monolayers, no tristearin was formed in microemulsions. Performing TLC on this microemulsion sample also confirmed the presence of these products.

4.4 Discussion

Although the enzyme is 1,3 specific, the presence of triglyceride was observed in the monolayer products but not in the microemulsions. This was mainly due to the long residence time of the products and substrate at the air/water interface in monolayers that favored acyl migration. It is likely that the products and substrates were relatively more stationary at the air/water interface of monolayers than at the oil/water interface of microemulsions. The enzyme presumably maintained its specificity in the microemulsions as well as in monolayers.

It has been shown [188] that lipases are able to catalyze synthesis reactions under conditions of "water starvation," which was confirmed using suspensions of the enzymes in organic solvents [21]. Other researchers have attempted to synthesize glycerides from glycerol and fatty acids in microemulsions with very low water content [120,144]. While no synthesis reaction was observed in microemulsions with water/glycerol ratio above 1:1 mole/mole [177]. We found that the same synthesis reaction was possible in monolayers with a water/glycerol ratio of 4:1, mole/mole (1:1, v/v). In fact a significant increase in surface potential (−60 mV) was observed with a water/glycerol ratio of 12:1, mole/mole, indicating the synthesis in monolayers. This was due to the orientation of the polar group of the fatty acid towards the active site of the enzyme and high concentration of fatty acid molecules near the active site. The concentration of fatty acid per unit interfacial area in monolayers was much higher than the interfacial concentration of fatty acids in microemulsions. Tristearin and monostearin were the main products from the reaction in monolayers, whereas distearin and monostearin were the main products from the reaction in microemulsions. The results obtained from the reaction in the microemulsion was consistent with the results obtained by Hayes and Gulari [177]. They used tetradecanoic acid as fatty acid and lipase from *R. delemar*. The presence of triglyceride in reactions in monolayers is mainly due to the residence time of reactants/products at the interface which was very long in case of monolayers as compared to that in microemulsions, and favorable orientation of stearic acid molecules in monolayers. The most interesting part of the synthesis in monolayers was the absence of distearin. The molecular size of lipozyme is 71.6 Å x 75.0 Å x 55.0 Å. Reactions in a monolayer were carried out at the surface pressure of 20 dynes/cm which gave an area/molecule of stearic acid at the air-water interface of about 25 Å^2/molecule. This gave a high probability of acyl migration from 1(3) position to 2 position and further esterification at 1(3) position. Therefore, triglycerides were formed. The acyl migration was a serious problem in monolayer as mentioned by Deveer et al. [178]. In the case of microemulsions, as distearin was formed at the interface, it diffused into the oil phase due to its partitioning behavior. It should be noted that these monolayers do not contain AOT at the air/water interface, so chemical compositions of interface in the microemulsion and in the monolayer are quite different. In order to elucidate the real cause of triglyceride formation in the monolayer, one needs to have the same interface composition for both monolayer and microemulsion interfaces. Chapter 5 explore the above question further.

4.5 Kinetic Analysis of Esterification Reactions in Water-in-Oil Microemulsions

It is well known that lipase is activated at an oil-water interface and exerts its hydrolytic activity only on those substrate molecules that are present at that interface. This makes the study of enzyme kinetics of lipolysis very complicated [189]. It has been found that the rate of hydrolysis depends on the triglyceride concentration at the interfacial area rather than on that in the bulk oil phase. In practice, this means that the measurement of lipase kinetic parameters in microemulsion systems is highly affected by the size of the microemulsion droplets and the presence of surfactants. To increase the reliability of the lipase kinetic parameter measurements, the literature cites several approaches, all emphasizing the importance of the interfacial area. Amongst these are the use of monolayers of substrate [189,190], the use of light scattering techniques to continuously monitor the interfacial area [191], and the use of stabilized reversed micellar solutions [192,120,193,194]. Due to the diameter of the reversed micelles, which is typically from 3 to 10 nm [120], an optically transparent and stable solution is obtained with such a large total interfacial area. This means the availability of substrate to the enzyme is no longer rate-limiting. Other researchers have found that the kinetics of reactions catalyzed by enzymes entrapped in such reversed micelles, as a rule, obey the classical Michaelis-Menten equations [102,133]. It is assumed thet the enzyme E and substrate S combine to form a complex ES, which then dissociates into product P and free enzyme E.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

$$ES \xrightarrow{k_2} E + P$$

The Michaelis-Menten rate expression is

$$V = V_{max}S/K_m+S$$

$$\text{where } V_{max}=k_2e_o \text{ and } K_m=(k_{-1}+k_2)/k_1$$

By rearranging this Michaelis-Manten equation, one can obtain the following expression,

$$1/V = 1/V_{max}+(K_m/V_{max})(1/S)$$

The plot of 1/V vs 1/S, known as a Lineweaver-Burk plot, separates

dependant and independent variables for the present study (Figure 4.4)
When v is equal to $v_{max}/2$ then S is equal to K_m

Han and Rhee [133] proposed a simplified model based on Michaelis-Menten kinetics to simulate the hydrolysis of olive oil in which only one substrate, triglyceride, and one product, fatty acid, were assumed. Based on this model for the present study, the initial rates of oleic acid consumption at a substrate concentrations ranging from 0.2303 M to 1.0948 M were calculated to execute the Lineweaver Burk plot. The linearity of the Lineweaver-Burk plot shown in Figure 4.4 suggests that the Michaelis-Menten model of enzyme kinetics holds for the lipase catalyzed esterification reaction in reversed micelles in the substrate range tested. The V_{max}, K_m, and K_2 values are 25 mM.min^{-1}, 3333 mM, and 111.2 min^{-1}. Since we don't know of any previous study on the kinetics of the esterification of fatty acid with glycerol, it is very difficult to compare the kinetic values found with literature data. For a non-specific lipase (from *Candida rugosa*) K_m and K_2 values are 717 mM and 67.1 μmol.min^{-1}.mg^{-1} were found by Tsai and Chiang [194] for the hydrolysis of olive oil by *Candida rugosa* lipase. Han and Rhee [120], on the other hand, found a K_m of 32 mM, the difference is possibly due to a different source or *Candida rugosa* strain of the lipase used. The K_2 value (111.2 min^{-1}) obtained for esterification reaction is somewhat higher than the value obtained by Derkensen et al for hydrolysis of crambe oil by *Mucor miehei* lipase (K_2 = 69.7 min^{-1}) [195]. The very high value of K_m (3333 mM) found indicates that the ES complex tends to break at a much faster rate than the rate of formation of the ES complex.

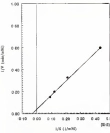


Figure 4.4 Lineweaver-Burk plot for enzymatic reactions in water-in-oil microemulsions of the present study

In summary, our results indicate that enzymatic synthesis in monolayers is indeed possible at higher water content as compared to microemulsions. Monolayers are more favorable for percent conversion of fatty acid into glycerides as compared to other systems such as microemulsions or aqueous solutions. Moreover, the concentration of the substrate at the air-water interface can be varied with surface pressure in monolayers. We also observed that mono- and triglyceride were the major products in monolayers, whereas mono- and diglyceride were the predominant products in microemulsions.

The kinetic analysis of the esterification reaction in the water-in-oil microemulsions indicates that this reaction was first order and it obeyed-Michaelis-Menten equation. The V_{max}, K_{mact} and K_2 values were 25 mM min^{-1}, 3333 mM and 111.2 min^{-1}. The very high value of K_m indicates that the ES complex tended to disappear much faster than it was formed.

CHAPTER 5
ESTERIFICATION IN Na-OLEATE STABILIZED MICROEMULSIONS AND MONOLAYERS

5.1 Introduction

Enzymatic catalyzed lipid transformations in water-in-oil microemulsions have been under study for the last decade [124,171,186 196] Lipase catalyzed hydrolysis of triglycerides to monoglycerides or all the way to glycerol and fatty acids, depending on the specificity of the enzyme, provides in high yields Lipase catalyzed glycerolysis seems to be a useful way of synthesizing monoglycerides. However enzymatic synthesis of triglycerides from glycerol and fatty acids has not been carried out successfully in microemulsions [136,144,197] Recently, we have shown that triglyceride synthesis could be performed at the air-water interface using a monolayer of fatty acids [198] or foam as the reaction medium [199] Since reactions in microemulsions and monolayers are both interfacial processes, it seemed to be of interest to investigate why triglycerides can be synthesized in monolayers but not in microemulsions This chapter aims at elucidating this issue

The lipase catalyzed condensations of fatty acid and glycerol in microemulsion systems studied so far have been performed in the presence of AOT or some other amphiphile which does not take part in the enzymatic process Hence, the interface between oil and water domains in these studies has largely consisted of surfactant molecules. For reaction to occur, the enzyme and the oil soluble reactants must be able to squeeze their way into the palisade layer. The situation is different for the monolayer experiment in which the fatty acids align at the air-water interface and there is no surfactant palisade layer to prevent the contact with the enzyme residing in the subphase.

This work aims at comparing lipase catalyzed condensation of fatty acid and glycerol in monolayers and in water in-oil microemulsions. The microemulsion palisade layer will consist of the same fatty acid and fatty acid soap molecules that constitute the monolayer at the air-water interface. The effect of the nonreactive surfactant on the reaction in the microemulsion is consequently eliminated.

In this experiment the microemulsion is stabilized by sodium oleate (Figure 5.1) instead of AOT as reported earlier [198] to compare mechanism involved in the monolayer and microemulsion. Here same weight ratio of oleic acid and sodium oleate is taken in both the systems. This experiment is designed to have an interface of the same composition of oleic acid and sodium oleate in both the monolayer and the microemulsion. Interestingly, the microemulsion containing sodium oleate as surfactant and tertiary butanol as co-surfactant can solubilize high amount of dispersed phase (45-50 %). Tertiary butanol was essential for formation of microemulsions. There was no tertiary butanol in the monolayer experiment.

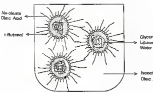


Figure 5.1 Schematic representation of Na-oleate, oleic acid and t-butanol stabilized w/o microemulsions.

5.2 Experimental Procedure

5.2.1 Monolayer Formation

The mixture of oleic acid and sodium oleate at 70:30, w/w, was dissolved in a solvent of hexane:methanol:chloroform 3:1:1, v/v, then it was spread on a subsolution of water and glycerol (1:1, v/v) using an Agla micrometer syringe. After the evaporation of the solvent, the monolayer was compressed with a waxed glass bar up to a desired initial surface pressure. When the surface pressure had reached a steady value, lipozyme was injected under the monolayer and stirred gently by rolling a magnetic bar to assure uniform distribution of the enzyme in the subsolution. The amount of enzyme was 70 LU per gram of the subsolution. The surface pressure and surface potential were measured after 30 seconds of stirring. After three hours, the monolayer was removed by suction and the product was dissolved in a solvent of methanol:chloroform 2:1, v/v. The sample for HPLC analysis was taken from the methanol and chloroform solvent phase.

5.2.2 Formulation of Microemulsion

The mixture of oleic acid and sodium oleate (70:30 w/w) was heated to 50°C and than cooled to room temperature to assure a homogenous mixture. Isooctane containing 48% tertiary butanol was mixed in various ratios with the mixture of oleic acid and sodium oleate. Then, those turbid mixtures containing the above four chemicals were titrated with dispersed phases. The turbid mixture became clear after the addition of a small amount of dispersed phase and it became again turbid after the further addition of dispersed phase. Turbidity was visualized using a cross polarizer in a constant temperature bath. The minimum amount of dispersed phase required was taken as the starting point of the L_2 phase. The maximum amount of dispersed phase before becoming turbid was taken as end point of the L_2 phase. Three dispersed phases, pure glycerol, mixture of glycerol and water and pure water were taken to determine L_2 phases. A pseudo ternary phase diagram representing L_2 phase, with dispersed phase as glycerol and other two components isooctane plus tertiary butanol and oleic acid plus sodium oleate was obtained. Similarly, a pseudo ternary phase diagram representing L_2 phase, with dispersed phase as glycerol plus water and other two components isooctane plus tertiary butanol and oleic acid plus sodium oleate, was obtained. Similarly, pseudo-ternary phase diagram representing L_2 phase, with dispersed phase as water and other two components isooctane plus tertiary butanol and oleic acid plus sodium oleate, was obtained. These phase diagrams show the increase in the solubility of dispersed phase with the increase in water concentration.

A separate technical phase diagram was prepared with glycerol and 9.1% water. The various compositions of microemulsions with 9.1% water were taken for self-diffusion measurements and esterification reaction. The microemulsions for esterification were left overnight to stabilize. The next day, fixed amount of lipozyme (50 LU/g of microemulsion) were added to various microemulsion samples and reactions were monitored with KOH titration

5.2.3 Self-diffusion Measurements

Self-diffusion coefficients were obtained using the ^{1}H-NMR Fourier-transform-pulsed-gradient spin-echo (FT-PGSE) technique using a standard JEOL FX-100 NMR spectrometer operating at a proton frequency of 99.6 MHz and a temperature of 25 ± 0.5°C. The PGSE measurements were taken by varying the duration of the gradient pulse (1 Gauss cm^{-1}) at a constant measuring time of 140 ms [200]

5.2.4 Reaction to be Investigated

The synthesis reaction taking place in the microemulsion and monolayer is,

$$Glycerol + Fatty\,Acid \xrightarrow{Lipase} \left[\begin{array}{l} Monoglyceride \\ Diglyceride \\ Triglyceride \end{array}\right] + Water$$

5.2.5 Monitoring the Reaction Using Titration, HPLC and TLC

The synthesis reaction in the microemulsion was monitored by measuring the amount of fatty acid consumed using titration with 0.1 KOH in ethanol and a sample without Lipozyme as a blank. The results were calculated as the percent conversion of fatty acid into glycerides.

To monitor the synthesis reaction, normal phase HPLC on a silica column (10 mm x 2.4 mm) with a UV absorbance detector of 213 nm cut-off wavelength and 0.05 absorbance units full-scale (aufs) was used to separate monoglyceride, diglyceride, triglyceride and fatty acid. A mixture of iso octane and iso propanol 94:6 v/v, was used as the mobile phase. The flow rate of the mobile phase was kept at 1 ml/min.

All analyses were done at a room temperature of approximately 22°C. The integrator plotted raw signal from the detector and determined the presence of peaks.

The TLC plates were run twice in diethyl ether up to 2 cm, and then in a hexane/diethyl ether/acetic acid mixture of 70:30:1, v/v/v

5.3 Results

5.3.1 Reactions in Monolayers

A monolayer of oleic acid and sodium oleate (70:30 w/w) was spread on a subsolution of glycerol and water (1:1, v/v, total volume 85 ml at pH 6.8). The monolayer was compressed to the desired surface pressure (18 dynes/cm) and Lipozyme was injected under the monolayer. Lipozyme was gently mixed under the monolayer. The surface pressure and surface potential were measured with time as described earlier [145]. It is evident from the data shown in Figure 5.2 that surface pressure increased by 5 dynes/cm whereas surface potential increased by about 120 mV. The synthesis reaction was carried out at 80 LU per ml of subsolution. There was an increase in the surface pressure of 5 dynes/cm, unlike the previous observation (Figure 4.1) where surface pressure remained unchanged after the addition of the Lipozyme under the monolayer of stearic acid [145]. It war mainly due to the larger area/molecule of oleic acid/sodium oleate as compared to the stearic acid which allowed penetration of the enzyme into the monolayer. On the other hand cleavage of the ester bond in lecithin monolayers decreases the surface

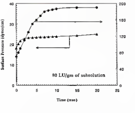


Figure 5.2 Surface Pressure/Surface Potential - Time curves for esterification of glycerol with oleic acid in monolayer.

potential [145,184]. We measured the change in surface potential of the monolayer at various initial surface pressures in order to elucidate the effect of surface pressure on the synthesis reaction. It is evident from the data presented in Figure 5.3 that an initial surface pressure of 18 dynes/cm was optimum for maximum change in the surface potential (i.e. maximum conversion). Initially the change in surface potential increased with an increase in the surface pressure. This was mainly due to the increase in the two dimensional concentration of reactant at the air/water interface. After the optimum surface pressure of 18 dynes/cm was reached, the change in the surface potential decreased with an increase in initial surface pressure because oleic acid and sodium oleate molecules were so tightly packed that the reaction was reduced.

The product from the monolayer was analyzed to confirm the synthesis reaction. After completion of the reaction, the monolayer was removed and mixed with a solvent of chloroform and methanol 1:2, v/v, to dissolve any mono-, di-, or triglycerides. An HPLC analysis of this sample indicated the presence of triolein (23%), diolein (19%) and oleic acid (58%) in the monolayer (Table 5.1). The degree of conversion of oleic acid was 41%. The TLC of the extracted monolayer also confirmed the presence of these products (Figure 5.4 and 5.5). Figure 5.4 and 5.5 showed the presence of triolein, 1,2 diolein, and 1,3 diolein from pure oleic acid monolayer and mixture of oleic acid and sodium oleate (70:30, w/w). There was no evidence of monoolein in the samples from the either monolayer.

The reaction described above was repeated, but now without sodium oleate.

Table 5.1 HPLC analysis of the products formed after 30 minutes of reaction in monolayers and microemulsions system.

System	Monoglyceride wt %	Diglyceride wt %	Triglyceride wt %
Monolayer based on oleic acid and sodium oleate	0	19	23
Monolayer based on oleic acid	0	10	31
Stearic acid monolayers	55.7	0	32.6
Microemulsion 5 based on oleic acid and sodium oleate	31	4	0

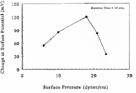


Figure 5.3 Effect of initial surface pressure on the change in surface potential in 1 hr.

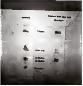


Figure 5.4 TLC of the standard and products from oleic acid monolayers.

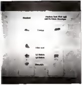


Figure 5 5 TLC of the standard and products from oleic acid and Na oleate monolayer

The monolayer consisted only of oleic acid. After 30 minutes the monolayer was analyzed by HPLC. As shown in Table 5.1, the amount of triolein was higher as compared to the reaction, with both oleic acid and sodium oleate present in the monolayer. The conversion of oleic acid was the same, approximately 40%.

In both cases, the reaction seems to reach equilibrium but in the first case the presence of sodium oleate decreased the formation of triolein. This could be an indication that the interaction of lipase with the monolayer is weaker in the system containing sodium oleate. It has been observed previously that adsorption of lipase to a monolayer is weaker with a more negatively charged interface [201].

When the enzyme was added to an aqueous subsolution without glycerol, that had a monolayer with a surface pressure of 18 dynes/cm, the surface pressure increased by 3 dynes/cm and the surface potential increased by 5 mV (data not shown). The increase in surface pressure without glycerol in the subsolution can be seen as an indication of the lipase being surface active [201].

5.3.2 Phase Diagrams and Reactions in Microemulsions

Figure 5.6, a pseudo-ternary phase diagram, represents L_2 phase with dispersed phase as glycerol and other two components are isooctane plus tertiary butanol and oleic acid plus sodium oleate. Figure 5.7, a pseudo-

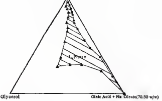


Figure 5.6 Phase diagram of glycerol-in-oil microemulsion.

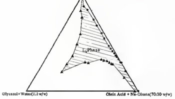


Figure 5.7 Phase diagram of glycerol and water (1:1 w/w) dispersed microemulsion.

ternary phase diagram, represents L_2 phase with dispersed phase as glycerol plus water and other two components are isooctane plus tertiary butanol and oleic acid plus sodium oleate. Figure 5.8, a pseudo-ternary phase diagram, represents L_2 phase with dispersed phase as water and other two components are isooctane plus tertiary butanol and oleic acid plus sodium oleate. As can be seen, the isotropic L_2 phase is relatively large in all systems. These phase diagrams show the increase in the solubility of the dispersed phase with an increase in water concentration. Without the cosurfactant t-butanol no L_2 phase was obtained for any water-glycerol mixture combined with the hydrocarbons heptane, isooctane or nonane. The cosurfactant tertiary butanol was chosen because it is not a substrate for lipase. Figure 5.9 is the pseudo-ternary phase diagram of the glycerol microemulsion with 9.1% water. The various components from this microemulsion shown in Table 5.2 were taken for self diffusion measurements and esterification reactions. The microemulsions for esterification were left overnight to stabilize. The next day, a fixed amount of Lipozyme (50 LU/g of microemulsion) was added to various microemulsion samples. The titration results show esterification of up to 35% in the microemulsion having high glycerol concentration (Figure 5.10).

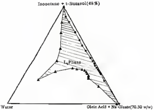


Figure 5.8 Phase diagram of water-in-oil microemulsion.

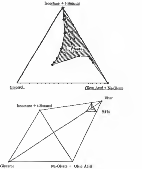


Figure 5.9 Phase diagram of glycerol and 9.1 % water dispersed microemulsion.

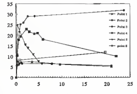


Figure 5 10 Percent conversion of oleic acid with respect to time

Table 5.2 Compositions (w/w) and self-diffusion coefficients for water (D_w) for microemulsions used. D^o_w is self diffusion coefficient of neat water (2.1×10^{-9} m^2sec^{-1})

System	Isooctane + t-Butanol (52:48) %	Oleic acid + Na-oleate (70:30) %	Glycerol %	Water %	D_w m^2S^{-1}	D_w/D^o_w
Sample 1	77.3	9.7	3.9	9.1	0.38	0.20
Sample 2	59.1	22.7	9.1	9.1	0.16	0.08
Sample 3	50.0	29.2	11.7	9.1	0.12	0.06
Sample 4	31.8	42.2	16.9	9.1	0.04	0.02
Sample 5	31.8	27.3	31.8	9.1	0.04	0.02
Sample 6	24.0	67.3	4.8	3.9	0.07	0.04

5.4 Discussion

There are very interesting results from the above experiments in monolayers and microemulsions. Compared to the monolayer experiments, the degree of conversion in microemulsions was generally low. There is a optimum glycerol to water ratio which gives a maximum degree of conversion in microemulsions. However, it has been seen that a very high glycerol to water ratio leads to poor lipase activity, possibly due to dehydration of the enzyme [143]. Most likely, there exists an optimum ratio between the two polar components like that seen in the previous Chapter 4 with the AOT microemulsion system. Some reactions exhibited a pronounced maximum in the degree of conversion with time. Lipase catalyzed lipid transformations that proceed via a maximum in yield at a certain time have been experienced before [143].

Self-diffusion NMR measurements of water were performed on the six starting compositions used for the enzymatic reactions. The results are included in Table 5.2. As can be seen from Table 5.2, the D-value of water in the series of samples with constant water content (samples 1-5) decreases with an increasing ratio of glycerol to water as well as sodium oleate plus oleic acid and water. It is also interesting to note that composition 6, which has the smallest content of both water and glycerol, does not give the lowest value of D_w/D^0_w. It seems that an increased glycerol content favors closed w/o structures. From the values of D_w/D^0_w it is obvious that all samples, possibly with the exception of composition 1, can be regarded as true w/o structures.

Some reactions show maximum conversion after a certain time. After maxima conversion goes down. One possible reason may be the microstructural change during the reaction. A reaction using composition 4 was monitored by self-diffusion NMR. Samples were taken from reacting system after 0.07, 4, and 24 hours and the D_w values were recorded. The results shown in Table 5.3 indicate that there are no major changes occurring in the microstructure of the microemulsion during the course of the reaction. A small decrease in the diffusion rate for water was observed after 4 hours of reaction time.

Table 5.3 Self diffusion coefficient of water (D_w) for microemulsion 4 during the course of the reaction. $D^o_w = 2.1 \times 10^{-9}$ m^{2}sec^{-1}

Reaction time (hr)	D_w/D^o_w
0.07	0.022
4	0.018
24	0.021

Microemulsions 3 and 4 have high concentration of oleic acid and sodium oleate as compared to microemulsion 1, 2, and 5. This would indicate that two reactions are taking place in this microemulsion system. The esterification of glycerol and oleic acid gives monoglycerides, diglycerides, and water. The water formed during esterification reacts with sodium oleate to give oleic acid and sodium hydroxide. When sodium oleate concentration was low, there was equilibrium between the esterification reaction and the secondary reaction. When concentration of sodium oleate was greater in the microemulsion (such as in 3 and 4), a large quantity of oleic acid was formed as a result of the water formed during esterification. The large amount of oleic acid resulted in substrate inhibition and, therefore, titration showed a decrease in the percent conversion of oleic acid. It has been observed that removal of water by the controlled addition of molecular sieve increases esterification [56]. Sodium oleate was used in this microemulsion to remove water formed during the esterification.

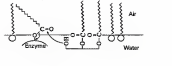
The low degree of conversion found in all the microemulsion reactions is probably due to the relatively high water content of the systems. A much higher ratio of glycerol to water should be favorable from an equilibrium standpoint. We have seen in Chapter 4 that lipases exhibit low activity at high water content. An interesting observation is that the reactions in a monolayer give a much higher oleic acid conversion (40-41%) than microemulsion 3 (6%), which had same glycerol to water ratio (1.2:1, w/w) as the monolayer subphase. Evidently, the special orientation of the reactant at the air-water interface enhances the normal esterification equilibrium. The increased residence time, closer molecular packing, and more favorable orientation of glycerol and fatty acid at the air-water interface also contributes to the high conversion in the monolayer as compared to the microemulsions, where fatty acid as well as mono- and diglyceride molecules have the freedom to partition into the oil phase.

Table 5.1 shows the results from the HPLC analysis after 30 minutes of reaction in the microemulsion system. As can be seen, monoolein and diolein are produced but no triolein. This is very different from the monolayer experiment in which 23% (w/w) triolein and 18% (w/w) diolein were produced, but no monoolein. Evidently, there is a fundamental difference between microemulsions and monolayers as reaction media for triglyceride synthesis, and this difference is not caused by the microemulsion surfactant (as no surfactant besides the substrate was used in these experiments). The main reason why triglycerides do not form in the microemulsion milieu is that the diglyceride is too lipophilic and has too low surface activity to stay at the oil-water interface. Once formed, diglyceride will partition into the hydrocarbon domain, whereas diglyceride remains at the air-water interface in the case of the monolayer experiments. The results obtained from this work tell us that the disappearance of the diglyceride from the interface is a rapid process compared with lipase catalyzed esterification to form triglyceride. Also, the monoglyceride formed is a lipophilic species and will partition mainly into the hydrocarbon domain and explains the low yield of diglycerides in the microemulsion experiments

The monolayer situation is very different Diglyceride formed has no other choice than to stay at the air/water interface. The suggested difference in interfacial behavior is shown schematically in Figure 5 10 In addition, the static nature of the air/water interface must constitute an ideal reaction zone for triglyceride synthesis because of acyl migration from 1(3) position to 2 position and further esterification at 1(3) position. Formation of three consecutive ester bonds to glycerol at this interface must be a much less complicated event than the corresponding process at the highly dynamic interfaces in microemulsions As seen in Chapter 4, the HPLC analysis of samples from stearic acid monolayers indicated the presence of tristearin (32.6%), Monostearin (55 7%), and stearic acid (11 7%), whereas sample from microemulsion indicated the presence of monostearin (12.4%), distearin (21 0%), and stearic acid (66 6%) In contrast to stearic acid monolayers, no tristearin was formed in microemulsions. It is mainly due to the dynamic nature of oil/water interface of microemulsions The absence of distearin in the product from stearic acid monolayer is due to very low area per molecule of stearic acid as compared to the area per molecule of oleic acid, which favored more acyl migration in case of stearic acid monolayer

In summary, our results indicate that enzymatic synthesis in monolayers is indeed possible at high water content as compared to microemulsions

The monolayer situation:



The microemulsion situation:

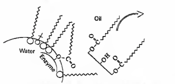


Figure 5 11 Schematic representation of reactions at the air/water interface and the oil/water interface

Monolayers are more favorable for percent conversion of fatty acid into glycerides as compared to other systems such as microemulsions or aqueous solutions Moreover the concentration of the substrate can be varied with surface pressure in monolayers. We also observed that di- and triglyceride were the major products in oleic acid monolayers, whereas mono- and diglyceride were the predominant products in microemulsions This new formulation of microemulsion can solubilize 45 50% of the dispersed phase and almost 20% of the oil-soluble substrate (fatty acid) Therefore, it has great potential for industrial application because major draw back for AOT stabilized microemulsions is very low oil-soluble substrate concentration.

CHAPTER 6
ESTERIFICATION IN FOAMS

6.1 Introduction

As we saw in Chapters 4 and 5, the synthesis in monolayers is indeed possible, but this route is good only for studies on the laboratory scale. Although monolayers and foams have the same air/water interface, foam has a very large interfacial area as compared to the monolayer. So, synthesis in foams has the potential for commercialization. In addition, foam is a solvent-free and surfactant or cosurfactant free system, so it overcomes various limitations imposed if the products are intended for use as food, cosmetic, or pharmaceutical additives. Nowadays attempts have been made to develop environmentally safe processes for the production of fine chemicals. To that end Kim et al. [202] have reported enzymatic synthesis by dispersion of capric acid and water in a surfactant free system. In this surfactant-free system, a mixture of capric acid, glycerol, and water was agitated with a magnetic stirrer in the presence of lipase. The products obtained by this system were similar to those obtained by the water-in-oil microemulsion system, i.e., large amounts of diglyceride and small amounts of monoglyceride and triglyceride. As they increased the chain length of the fatty acid, the yield of products from aqueous dispersion decreased dramatically [202].

6.2 Experimental Procedure

Because enzymatic synthesis in foam is a solvent and surfactant-free system, it is also an environmentally safe process. With its large interfacial area, foam is ideal for the esterification of glycerol and fatty acid. In this study, stearic acid was used as the foaming agent as well as a reactant. A glass column was filled with a mixture of 30 ml of double distilled water and glycerol (1:1 v/v). Stearic acid (0.2 g, 99% pure) was dissolved in 4 ml of chloroform, then added into the column. Later it was realized that there was no need of addition of chloroform. Foam can be generated by the direct addition of stearic acid. Air was blown at a high flow-rate for 15 minutes to evaporate all the chloroform before the addition of lipase. This process avoids lipase inhibution by chloroform [203]. A 0.2 ml solution of Lipozyme was added into the column. The enzyme catalyzes the esterification reaction as well as increases the foam height in the column. Air was blown continuously during the reaction at a very low flow rate to maintain a large foam volume (Figure 1.6). Samples were taken during the reaction, and mixed immediately with a mixture of iso-octane and 2-propanol (4:1, v/v) in order to stop the reaction.

6.3 Results and Discussion

A normal phase HPLC was used to analyze the reaction products at various time intervals. The silica column and the mixture of iso-octane and 2-propanol (96:6, v/v) as eluent were used at room temperature. The results are shown in Figure 6.1. The reaction performed in foam produced large amounts of triglyceride

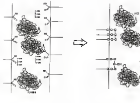


Figure 6.1 Schematic representation of thin film of foam before and after reaction.

and diglyceride, 20% and 55% (by weight), respectively. This result is similar to that found in esterification in monolayers in Chapter 5, whereas products from the water-in-oil microemulsions are monoglyceride and diglyceride. The differences in products from the three media, namely foams, monolayers, and microemulsions, can be explained on the basis of the two phase model proposed by Verger [189]. In this model, the first step is the penetration of the water-soluble enzyme into the monolayer. The second step is the formation of an enzyme-substrates complex at the air/water interface. In the catalytic step, the product is formed and the enzyme is regenerated. If the product is water soluble, it will migrate into the aqueous phase, in the case of foam into the thin film of foam. If the product is oil soluble the product will remain at the interface, in the case of the air/water interface, resulting in acyl migration from the 1(3) to the 2 position and further reaction at the 1(3) position (in case of both foams and monolayers) resulting in the formation of triglycerides. Acyl migration is a serious problem in monolayers [178]. Deveer et al. has used synthetic pseudoglycerides to avoid acyl migration for the kinetic study of lipase in monolayers [178]. On the other hand, the product will partition into the oil phase in the case of an oil/water interface (microemulsions). Figure 6.2 shows the expanded thin film before and after the esterification. The molecules of stearic acid at the interface make a complex with lipozyme and then react with glycerol to give diglycerides. In the very short run, most of the reaction products might be diglyceride because the enzyme is 1(3) specific, but in the long run, due to acyl migration, further reaction takes place and hence triglycerides are formed. Also, the

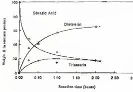


Figure 6.2 HPLC analysis of products from foam with time.

amount of diglyceride increases as the reaction time increases before the reaction reaches the equilibrium state as shown in Figure 6.1. We also carried out the reaction in the column without air flow. Because there was no significant change in the concentration of stearic acid after 3 hours, it appears that foaming is essential to produce diglyceride and triglyceride.

In summary, the esterification reactions in foam, which involve the air/water interface, yielded products in different proportions from those produced in water-in-oil microemulsions. In foam the reaction produced large amounts of diglyceride (55 wt%) as well as triglyceride (20 wt%), whereas the reaction in microemulsions produced large amount of diglyceride (50 wt%). Because the large air/water interface at which the reaction takes place, this reaction using foam could be employed for large scale production of di- and triglycerides by the enzyme without using solvents or surfactants.

CHAPTER 7
OXIDATION OF LIPID MONOLAYERS

7.1 Introduction

The oxidation of insoluble monolayers by aqueous potassium permanganate solutions has been studied by many investigators [204-211] by measuring simultaneously the time dependence of the apparent molecular area and the surface pressure. They found that as one spreads a monolayer on the aqueous permanganate solution, the oxidation of the monolayer and evaporation/solubilization of the solvent occur simultaneously, which affects the area/surface pressure at the very beginning of oxidation. Because of this, in the present study, the $KMnO_4$ solution was injected under the monolayer after compressing it to the desired surface pressure.

It has been reported [210] that the intermediate products formed during the cleavage of double bonds contain cis-epoxy groups. Because some of the products after oxidation are insoluble in water, the surface potential of the monolayer is considered to be a more reliable parameter to predict the rate of the oxidation reaction.

Oxidation of unsaturated monoglycerides, diglycerides, and triglycerides is responsible for rancidity in oil containing products. The effects of various monoglycerides and diglycerides on the oxidative stability of soybean oil has been investigated by rates of disappearance of headspace oxygen in purified oils containing

0.0, 0.25, and 0.5% monostearin, distearin, monolinolein or dilinolein [212]. The monoglycerides and diglycerides act as prooxidants in soybean oil, but the mechanism of this effect was not delineated. In this study, we present the effect of the number of double bonds and the initial surface pressure on the oxidation of monoglycerides (monoolein, monolinolein), diglycerides (diolein, dilinolein), and triglycerides (triolein, trilinolein).

7.2 Experimental

7.2.2 Methods

Lipid solutions were prepared in a mixed solvent system containing chloroform-methanol-hexane (1:1:3) of spectroscopic grade. The surface pressure and surface potential of lipid monolayers were measured on a 0.02 M NaCl subsolution at 25°C and a pH of 6-7 as described elsewhere [187]. At various initial surface pressures, 5 ml of $KMnO_4$ solution (1 mg/ml) in 0.02 M NaCl was injected under the monolayer, resulting in a final concentration of 12.5 μgm of $KMnO_4$ per ml of subsolution. Fresh subsolution (5 ml) was removed after injecting the $KMnO_4$ solution in order to maintain the same air gap for surface potential measurements. For proper mixing of the $KMnO_4$ in the subsolution, a magnetic bar was rolled along the length of the trough at slow speed. The surface tension and surface potential with respect to time were measured after carrying out the initial stirring.

The surface shear viscosities of these lipids were measured using a deep channel surface viscometer for the monolayers at a surface pressure of 10 dynes cm^{-1}

[213-216] In the deep-channel surface viscometer, the channel walls are stationary, concentric cylinders, the floor of the viscometer moves with a constant angular velocity. A small Teflon particle was placed at the interface to determine the center line velocity, than the time for the particle to complete a rotation was recorded. Each experiment was repeated five times and the average velocity of the particle was used to calculate the surface shear viscosity [215,216]. The surface pressure was measured in the surface viscometer at the beginning as well as at the end of the experiment to establish that the monolayer did not dissolve in the subsolution.

7.3 Results and Discussion

7.3.1 Oxidation of Monoolein and Monolinolein Monolayers

Figure 7.1 shows the π-Area and ΔV-Area curves for monoolein and monolinolein. The area per molecule of monoolein (26 Å^2) was greater than that of monolinolein (24 Å^2), which is consistent with their molecular structures and agrees with values in the literature [217]. Monoolein has a double bond at C_9 whereas monolinolein has two double bonds at C_9 and C_{12} positions. The second kink at the C_{12} position in monolinolein allows the molecule to cover less area as compared to monoolein as shown in Figure 7.2. Both the surface pressure and surface potential decreased with time in the case of monoolein and monolinolein after the addition of $KMnO_4$ solution (Figure 7.3 & 7.4). This is due to solubility of glycerol -1-o-nonanoic acid and short-chain fatty acids formed in monoolein and monolinolein.

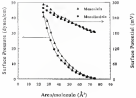


Figure 7.1 π-Area and ΔV-area curves of monoolein and monolinolein monolayers

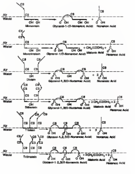


Figure 7.2 A schematic diagram of lipid molecules after oxidation at the air/water interface.

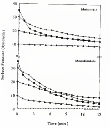


Figure 7.3 Effect of $KMnO_4$ solution on the surface pressure of monoolein and monolinolein monolayers

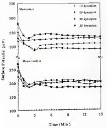


Figure 7.4 Effect of KMnO$_4$ solution on the surface potential of monoolein and monolinolein monolayers.

Both parameters decreased more sharply in the case of monolinolein compared with monoolein at the same initial surface pressure. This may be due to the solubility of short-chain acids produced by oxidation (malonic acid and hexanoic acid) in the monolinolein monolayer whereas nonanoic acid formed in the monoolein monolayer is less soluble than malonic acid and hexanoic acid. At higher initial surface pressure, the surface pressure and the surface potential decreased more rapidly (Figure 7.3 and 7.4). This is presumably due to the increased desorption rate of the short chain acids. These results show that monoolein and monolinolein react even at high surface pressure (35 dynes cm^{-1}). This may be due to the presence of the cis double bond, which give a high anchoring effect to the interface upon oxidation producing -OH groups

7.3.2 Oxidation of Diolein and Dilinolein Monolayers

The π-Area and ΔV-Area curves for diolein and dilinolein are shown in Figure 7.5. The areas per molecule of diolein and dilinolein are 53 Å^2 and 49 Å^2 respectively. In the case of dilinolein, when $KMnO_4$ solution is injected under the monolayer, the surface pressure increases (Figure 7.6) but its surface potential decreases (Figure 7.7) at low initial surface pressure. This means that oxidation is taking place but the product, glycerol-1,3-o-nonanoic acid, a product is not dissolving in the subsolution due to its two acyl chains, which results in an increase in surface pressure (Figure 7.2). However, the surface pressure and surface potential are almost constant when $KMnO_4$ solution is injected at a surface pressure of

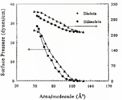


Figure 7.5 π-Area and ΔV-area curves of diolein and dilinolein monolayers.

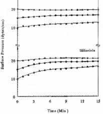


Figure 7.6 Effect of $KMnO_4$ solution on the surface pressure of diolein and dilinolein monolayers

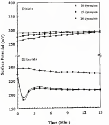


Figure 7.7 Effect of $KMnO_4$ solution on the surface potential of diolein and dilinolein monolayers.

20 dynes cm^{-1} indicating that there is no oxidation taking place at that surface pressure. This also suggests that dilinolein molecules are tightly packed at that surface pressure and the double bond is not accessible to the $KMnO_4$ in the water. If indeed this is the case, diolein should have not reacted at a surface pressure lower than for dilinolein because diolein has only two double bonds per molecule. It is evident from Figure 7.6 that at 10 dynes cm^{-1} there is a slight increase in surface pressure. The surface potential for diolein at an initial surface pressure of 20 dynes cm^{-1} is constant and the surface potential at an initial surface pressure of 10 and 15 dynes cm^{-1} increases and reaches at same surface potential, as is obtained when the film is at a surface pressure of 20 dynes cm^{-1} after 15 minutes. This means that the number of electric dipoles is the same for all monolayers regardless of their initial surface pressures. The results also suggest that the formation of cis-epoxy groups in diolein molecules at low surface pressures is taking place, which increases both the surface pressure as well as surface potential of the monolayer (Figure 7.6 and 7.7) [210]

7.3.3 Oxidation of Triolein and Trilinolein Monolayers

The areas per molecule of triolein and trilinolein are 80 Å^2 and 76 Å^2 respectively (Figure 7.8) [218]. For trilinolein, when $KMnO_4$ solution is injected under the monolayer at $\pi = 5$ dynes cm^{-1}, the surface pressure increased but the surface potential decreased slowly at low initial surface pressure. At high surface pressure ($\pi = 10$ dynes cm^{-1}), a similar trend was observed (Figure 7.9 and 7.10)

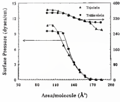


Figure 7.8   π-Area and ΔV-area curves of triolein and trilinolein monolayers.

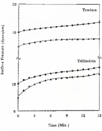


Figure 7.9 Effect of $KMnO_4$ solution on the surface pressure of triolein and trilinolein monolayers.

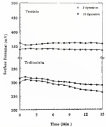


Figure 7 10 Effect of $KMnO_4$ solution on the surface potential of triolein and trilinolein monolayers.

Trilinolein oxidized and glycerol 1,2,3-o-nonanoic acid remained at the surface, this requires more area than for the trilinolein molecule due to three carboxylic groups as shown in Figure 7.2. At a high initial surface pressure, trilinolein molecules are squeezed out of the water surface due to the lack of polar groups providing anchoring on the water surface. Similar results are shown using triolein, but changes are less pronounced because triolein has only three double bonds per molecule as compared with six in trilinolein.

7.3.4 Surface Viscosity and Oxidation of Lipid Monolayers

The surface viscosity of lipid monolayers provides useful information about the interaction of polar groups with the subsolution as well as molecular interactions in the monolayer. The surface shear viscosity of the monolayer increases as the interaction (or anchoring) of the polar group with the subsolution increases (e.g. the surface shear viscosity of monoolein is higher than that of diolein (Table 7.1)). The surface shear viscosity also increases as the chain-chain interactions increase (e.g. dilinolein has a higher surface viscosity than diolein (Table 7.1). If we compare the surface shear viscosity (Table 7.1) with the $KMnO_4$ induced oxidation rate of monoglycerides (Figure 7.4), diglycerides (Figure 7.7) or triglycerides (Figure 7.10), it is evident that the presence of linoleic acid chains increases the surface shear viscosity as well as the oxidation rate of the glycerides, compared with the oleic acid chains. This supports our explanation that both the surface viscosity and the oxidation rate of the monolayers are a function of lipid structure and their interaction with each other as well as with the subsolution.

Table 7.1 Surface shear viscosity of lipid monolayers at a surface pressure of 10 dynes cm^{-1}.

Lipid	Surface shear viscosity at 25°C 10^{-4} P
Dilinolein	9.614
Monolinolein	7.447
Monoolein	6.149
Trilinolein	4.697
Diolein	3.405
Triolein	2.760

In summary, the results of these studies on monoglycerides, diglycerides and triglycerides indicate that the rates of oxidation of dilinolein and monolinolein monolayers are significantly high compared with those of the other lipid monolayers. Monoolein and monolinolein monolayers even react at high surface pressure (35 dynes cm^{-1}). Both the rate of the oxidation reaction in the monolayer and the surface shear viscosity of the monolayer are functions of the lipid structure and of their interaction with each other as well as with the monolayer. The oxidation rate based on ΔV and π were as follows

dilinolein > monolinolein > monoolein > trilinolein > diolein > triolein,

The surface viscosities of the monolayers of above lipids also were in the same order as those of the oxidation rate.

CHAPTER 8
CONCLUSIONS AND RECOMMENDATIONS FOR THE FUTURE RESEARCH

8.1 Lipase Catalyzed Esterification in Microemulsions

The synthesis reaction was found to be optimum at 0.64 mole fraction of glycerol to water because additional water shifts the equilibrium towards hydrolysis. The conversion increases with chain length (from hexane to hexadecane) after one hour but for equilibrium conversion after 10 hours the reverse is true. The conversion of fatty acids into glycerides increased with increased fatty acid chain length. A maximum equilibrium percent conversion at 1:1 molar ratio of oleic acid to glycerol was observed. A maximum conversion of 84% was found in the case of linoleic acid, 80% for linolenic acid, 71% for oleic acid, and 33% for stearic acid. The HPLC analysis showed the presence of monoglycerides and diglycerides but no triglyceride in the products from AOT stabilized water-in-oil microemulsions.

Because the new formulation of sodium oleate stabilized microemulsion can solubilize 45-50 % of the dispersed phase and almost 20% of the oil-soluble substrate, it has great potential for industrial application because it does not have the major drawback of AOT stabilized microemulsions, namely very low oil-soluble substrate concentration. Further research is needed to improve the esterification in this new microemulsion systems and various parameter should be checked to improve the reaction kinetics. Also, esterification reactions should be done in a microemulsion having large amounts of glycerol. The present microemulsion contained 9.1 percent water by weight. The amounts of water should be decreased in future microemulsions to shift the equilibrium favorable to the esterification. Other parameters like the amounts of t-butanol, the pH, and the temperature also should be monitored to improve reaction kinetics.

This microemulsion presents a new direction for organic reactions in microemulsions. For organic reactions having polar and nonpolar substrates, oil water interface of microemulsion could be stabilized with a nonpolar substrate or a salt of nonpolar substrate. This would remove the separation problem found in commonly used surfactants employed to form microemulsions.

The kinetics analysis of AOT stabilized microemulsion fits well to the Michaelis-Menten equation. A comparison of kinetic values of esterification reaction in AOT microemulsion with the esterification reaction in a microemulsion stabilized with sodium oleate/oleic acid could show the usefulness of this new microemulsion systems. A kinetic analysis of the reaction in sodium oleate stabilized microemulsion ought not fit into the Michaelis-Menten equation because the oil-water interface in this instance has large amounts of oleic acid, while, in the AOT stabilized microemulsion, oleic acid concentration at the oil-water interface is very low. Thus, the analysis of reaction kinetics in these microemulsions may delineate the nature of microemulsions in these two systems.

8.2 Lipase Catalyzed Esterification in Monolayers

Our results indicate that enzymatic synthesis in monolayers is indeed possible at higher water content as compared to microemulsions. Monolayers are more favorable for percent conversion of fatty acid into glycerides as compared to other systems such as microemulsions or aqueous solutions. Moreover, the concentration of the substrate at the air-water interface can be varied with surface pressure in monolayers. We also observed that mono- and triglyceride were the major products in stearic acid monolayers, and di- and triglyceride were major products from oleic acid monolayers, whereas mono- and diglyceride were the predominant products in microemulsions having stearic acid or oleic acid as substrate.

More detailed experiments are required to elucidate the esterification mechanism. Synthesis using monolayers as reported in this research opens a new field of study for other synthesis reactions in monolayers, which would mimic various kinds of synthesis in the human body. The results reported here show that the products from a monolayer system are different from those in a microemulsion. Various parameters, like the number of double bonds in a fatty acid, a mixture of saturated and unsaturated fatty acids and a mixture of cholesterol, or unsaturated fatty acid, should be studied for esterification reaction. The effect of enzyme on monolayers of monoglycerides and diglycerides having saturated and unsaturated fatty acid chains should be studied to delineate the acyl migration and esterification. These studies would correlate the effects of the state of the film (e.g. solid, liquid etc.) with the kinetics of esterification reaction. Similarly, the hydrolysis of saturated and unsaturated glycerides in the presence of cholesterol should be relevant to the behavior of fat in the human body

8.4 Esterification in foam

The esterification reactions in foam, which involve the air/water interface, yielded products in different proportions from those produced in water-in-oil microemulsions. In foam the reaction produced large amounts of diglyceride (55 wt%) as well as triglyceride (20 wt%), whereas the reaction in microemulsions produced large amount of diglyceride (50 wt%). The products obtained from a reaction in the foams were similar to those in monolayer, e.g., diglyceride and triglyceride. Because the large air/water interface at which the reaction takes place, this reaction using foam could be employed for large scale production of diglyceride and triglycerides by the enzyme without using solvents or surfactants.

Lipase catalyzed esterification reaction in foams is very promising. Reactions in foams open a new and potentially good route for commercial scale-up. A detailed study is needed to correlate various parameters such as the glycerol/water ratio, the glycerol/fatty acid ratio, the enzyme concentration and effect of time on final yields of products. The kinetic analysis of the reaction in foam would be very nice to compare with other systems like microemulsions, monolayers and aqueous micellar systems.

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